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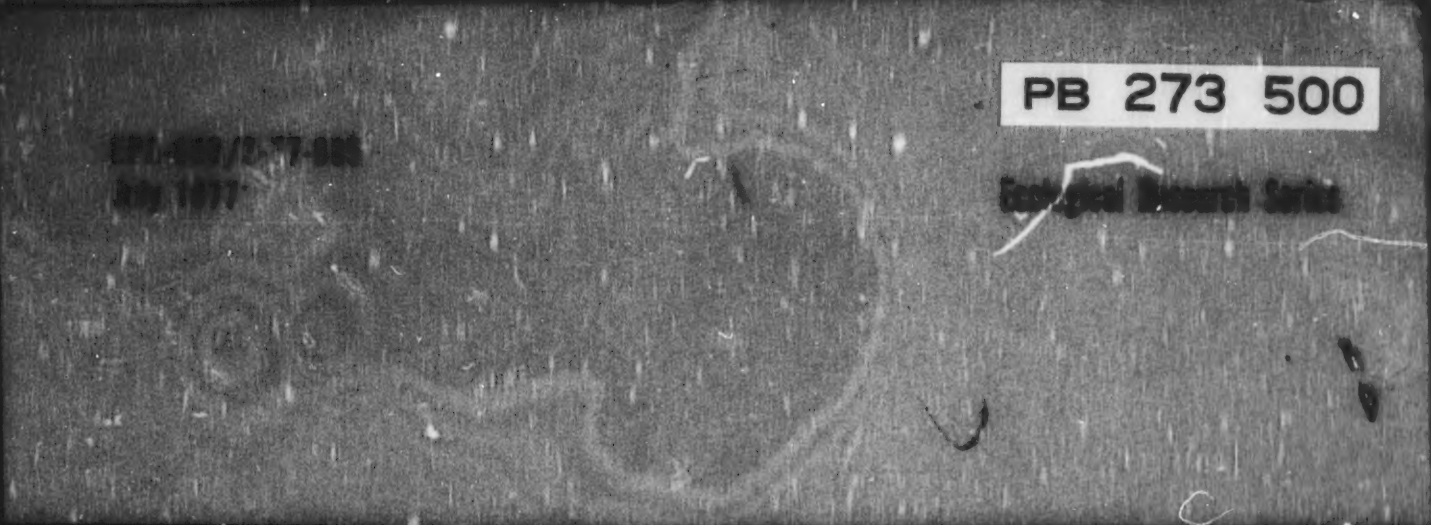

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RECENT ADVANCES IN FISH TOXICOLOGY

A Symposium

edited by

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sponsored by

Corvallis Environmental Research Laboratory
Corvallis, Oregon 97330

in cooperation with

Department of Fisheries and Wildlife
Oregon State University
Corvallis, Oregon 97331

CORVALLIS ENVIRONMENTAL RESEARCH LABORATORY
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FOREWORD

Effective regulatory and enforcement actions by the Environmental Protection Agency would be virtually impossible without sound scientific data on pollutants and their impact on environmental stability and human health. Responsibility for building this data base has been assigned to EPA's Office of Research and Development and its 15 major field installations, one of which is the Corvallis Environmental Research Laboratory (CERL).

The primary mission of the Corvallis Laboratory is research on the effects of environmental pollutants on terrestrial, freshwater, and marine ecosystems; the behavior, effects and control of pollutants in lake systems; and the development of predictive models on the movement of pollutants in the biosphere.

This report is a compilation of reports presented at the Symposium on Recent Advances in Fish Toxicology, January 13-15, 1977 in Corvallis, Oregon. The Symposium was cosponsored by The Corvallis Environmental Research Laboratory and the Department of Fisheries and Wildlife, Oregon State University.

A.F. Bartsch
Director, CERL

PREFACE

The symposium Recent Advances in Fish Toxicology was held in Corvallis, Oregon on January 13 and 14, 1977. The Corvallis Environmental Research Laboratory of the United States Environmental Protection Agency and the Department of Fisheries and Wildlife of Oregon State University cosponsored the symposium to encourage the rapid communication of recent findings between fish toxicologists. New legislation has increased the need for communication between fish toxicologists, and the 1976 Toxic Substances Act (PL 94-469) indicates a new era is beginning for water pollution control. The law now requires the clearance of new chemical products that might enter waterways before such substances are manufactured and sold. Prediction of the probable toxic effects to fish and other aquatic organisms must be based on a developing assessment methodology. Symposium participants attempted to summarize some of the recent findings in fish toxicology or pointed out the research that is needed to meet the new legislative mandate. The symposium is dedicated to Professor Peter Doudoroff who is concluding a long and active research and teaching career. His pioneer research with the Public Health Service and Oregon State University helped to define many of the physical and chemical conditions required for aquatic life. The results of his research have been applied by many countries to establish water quality standards. He has been generous in his advice and counsel and many of the symposium presentations were made by former students and colleagues.

Richard A. Tubb
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INTRODUCTORY REMARKS

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I want to begin my remarks with a quotation:

"To a far greater extent than ever before, we live in a man-created and man-controlled environment. It is within our power to shape our own future, to guide the evolving patterns of society and determine the nature of the surroundings in which we and our children will live.

". . .it might be helpful if I were to sketch out for you, in very broad strokes, the view of the water pollution problem from the national window of a federal agency charged with rather far-ranging responsibilities in this field.

"In doing so, I should like to develop four principal points:

"First, that water pollution control is an integral part of the broader problem of water resource development and use;

"Second, that water pollution control is an inseparable part of the broader problem of environmental health protection;

"Third, that an impressive amount of productive activity is already underway in controlling water pollution;

"And fourth, that the problem demands a still stronger effort on the part of federal, state, and local authorities, industries, and all others concerned."

These were remarks delivered by Dr. Leroy E. Burney, Surgeon General of the Public Health Service, in an opening address before the National Conference on Water Pollution held 16 years ago on December 12.

You may have noticed there was no plea for attention--scientific or otherwise--to pollution impacts on fish and other aquatic life. In fact, the existence of aquatic life was not even acknowledged. The same was true in the 30 recommendations that came from the conference.

At the time of that conference in 1960, Dr. Doudoroff had already been working as a pioneer in the field of water pollution biology for almost 20 years. I am sure, in those early days of the '40's, he was on a first-name basis with all of his colleagues in the United States. They numbered only a handful. It was a challenging time because the ground was fertile for plowing with much to be discovered. It was also a frustrating time because the place of biology and the role of biologists in water pollution control was neither well understood nor widely accepted. These conditions have changed.

Water pollution control did not become a national movement until 1949. Previously, a few progressive states were moving forward with vigor; others were standing still. Water pollution control programs, where they existed, consisted mainly of efforts to stop discharging untreated municipal sewage in order to protect human health and to terminate public nuisances. Perhaps some of you here today remember what the Willamette River, flowing through this city, was like in those days. Industry had no strong motivation to protect freshwater and marine organisms.

It is fitting that this symposium be dedicated to Dr. Peter Doudoroff. His past and continuing monumental contributions to the subject of fish toxicology have not only generated new knowledge that this nation needed but also served to train researchers and stimulate many others. It is appropriate at this time for a symposium to look at recent advances in fish toxicology. In this stock-taking, we should be mindful of the broader framework into which these efforts fit today. That framework has many aspects; two are especially notable.

One point is the growing frequency and severity of environmental crises that come largely from human ignorance, indifference and economic greed. We have just finished the worst year in our history of major oil spills (15 tankers and 200,000T). Crises involving mercury, PCB's, asbestos, and kepone are still fresh memories. The "Legionnaires' Disease" has us baffled. History will show that we and other nations have been ineffective in foreseeing the next environmental crisis.

The other aspect is more encouraging. Today the nonhuman side of environmental pollution has been acknowledged as important. During the last three fiscal years, EPA assigned from 83-89% of its research dollars to problems other than human health--much obviously in biologically-oriented areas. There is no action impinging on environmental quality that is not noticed by some powerful, national citizens' organization. And finally, laws to protect the environment are becoming stronger (unfortunately more complex) and more far-reaching. In the context of this symposium, one of the most important pieces of legislation may turn out to be the Toxic Substances Control Act (PL 94-469) signed into law on October 12, 1976.

The Act is 49 pages long. It is complex. Its far-reaching impact can be surmised somewhat from its policy statement. Let me quote--

"It is the policy of the United States that--

"(1) adequate data should be developed with respect to the effect of chemical substances and mixtures on health and the environment and that the development of such data should be the responsibility of those who manufacture and those who process such chemical substances and mixtures;

"(2) adequate authority should exist to regulate chemical substances and mixtures which present an unreasonable risk of injury to health or the environment, and to take action with respect to chemical substances and mixtures which are imminent hazards; and

"(3) authority over chemical substances and mixtures should be exercised in such a manner as not to impede unduly or create unnecessary economic barriers to technological innovation while fulfilling the primary purpose of this Act to assure that such innovation and commerce in such chemical substances and mixtures do not present an unreasonable risk of injury to health or the environment."

In the past, much of the bioassay development effort focused on lethal effects of toxic substances. Efforts today, and especially those responding to the Toxic Substances Control Act, will emphasize sublethal effects. Many of you may be involved in this activity. If you are, your work will be more effective and successful because of the foundation that Dr. Doudoroff and his colleagues have established.

A MULTIPLE APPROACH TO SOLVING THE GAS SUPERSATURATION PROBLEM

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ABSTRACT

Gas supersaturation of water was first recognized as a serious problem in the Snake and Columbia rivers of the Pacific Northwest. To solve the problem, a multiple approach was used combining laboratory and field studies to determine sources, effects, persistence, and prevention of the supersaturation. Classical bioassays were used to determine effect, but additional tests were needed because of the unique nature of supersaturation. These tests included assessment of avoidance capability of fishes, assessment of depth compensation and temperature effects, and field surveys of aquatic organism distribution in the affected areas. Data from the combined approaches were used to set safe levels for aquatic organisms. In addition, engineering expertise from other groups was applied in an attempt to prevent or mitigate the effects of supersaturation.

INTRODUCTION

The purpose of this paper is to demonstrate how a cooperative effort by a number of agencies was used in an attempt to solve the problem of gas supersaturation of water in the Columbia River Basin and other rivers and coastal waters of the U.S. The multiple approach combined classic toxicity studies, newly-designed special effect studies, field studies, and engineering expertise. The desire is not so much to present data on any particular part of the study as to chronicle the overall effort and to show how toxicity studies are an integral part of the problem definition and solution.

THE PROBLEM

Air supersaturation of water was first noted in hatchery and aquarium facilities in the early 1900's (Gorham 1901), and was ascribed as causing the condition in fish known as gas bubble disease. Based on Gorham's conclusions and the more precise analytical methods of Van Slyke and Neill (1924) for nitrogen determination, dissolved nitrogen gas was postulated to be the cause of gas bubble disease and received primary research emphasis. Supersaturation caused by entrainment of air in water spilled over dams first became a problem on the Columbia River when Bonneville Dam was constructed in 1938, although it was apparently undetected at that time. As more dams were constructed on the river the problem increased; water was supersaturated by each dam with the result that the entire river could be supersaturated during some periods of the year (Ebel 1969; Weitkamp and Katz 1973, 1975; Rucker 1972). Considering only the Snake River chinook salmon and steelhead stocks, Ebel et al. (1975) forecast a total loss of 2 million adult fish during the period 1976 to 2000 if no remedial actions were taken to reduce the hazards of supersaturation. In economic terms (1974 dollars), this loss would range between $47.2 and $126.9 million. These figures are very conservative since they do not take into account stock from the Columbia River and its tributaries, nor other species such as sockeye and coho salmon.

ORGANIZATION FOR SOLVING THE PROBLEM

As supersaturation on the Columbia and Snake rivers became more severe the U. S. Secretary of the Interior and the Tri-State Governors Conference (Idaho, Washington, and Oregon) formed a Nitrogen Task Force to work out solutions to the problem. The task force was made up of representatives from 23 public and private agencies. One of their first duties was to organize a division of labor between various agencies for research on the problem. The Environmental Protection Agency, represented by the Western Fish Toxicology Station, was detailed to carry out laboratory studies to determine safe levels of supersaturation for both adult and juvenile salmonids. This study was later expanded to include food organisms as well as predators and competitors of these fishes. The National Marine Fisheries Service and the states of Washington, Oregon, and Idaho were detailed to conduct field studies to determine effects and persistence of supersaturation in the Columbia River Basin. They also conducted some laboratory studies on effects.

The U. S. Army Corps of Engineers, in cooperation with the Bonneville Power Administration, were already operating the dams and regulating the flow on the lower river; their responsibility for flow manipulation and control of the power plants continued. In addition the Corps funded fisheries research through the National Marine Fisheries Service laboratories and conducted engineering and modeling studies to provide solutions to the problem.

The Northwest Utility Cooperative, together with public and private research organizations such as Parametrix, Inc., and Battelle Northwest, provided funding and cooperation for additional research (Weitkamp and Katz 1973, 1975).

DEVELOPMENT OF MEASUREMENT TECHNIQUES

At the beginning of the supersaturation study, measurement techniques had to be developed for use in both laboratory and field experiments. Previously two standard techniques had been employed, the standard Winkler determination for dissolved oxygen (APHA 1971) and the Van Slyke method for nitrogen determination (Van Slyke and Neill 1924). The oxygen determination method was quick and easily used, but did not determine nitrogen or total gas saturation levels. The Van Slyke method measured O_2, N_2 and total gas, but was relatively cumbersome and difficult for field use. A third technique, the gas chromatograph, was also available for determining nitrogen, oxygen, or carbon dioxide. It is relatively quick and precise but requires expensive equipment which is not readily portable (Fickeisen et al. 1975). The development of the Weiss saturometer in 1970 significantly changed the method of analysis, and made field and some laboratory determinations relatively easy to accomplish. The saturometer, developed by Ray Weiss (1970, unpublished data, Scripps Inst. Oceanog., La Jolla, Cal.) and adapted for field use by Robert Rulifson (S. Lambert and R. L. Rulifson, 1972, unpublished report, U. S. Environmental Protection Agency, Seattle), consists of a metal framework upon which is wound a length of about 100 ft (the length is variable) of semi-permeable, medical-grade, silastic tubing. The tubing is permeable to gas when submerged but is not permeable to water; thus, the gas in the water goes through the wall of the tubing until gas pressure within the water and the tubing becomes equilibrated. The gas pressure within the tubing is then measured by a manometer to determine the difference between gas pressure in the water and gas pressure in the atmosphere. With a known atmospheric pressure one can calculate percent total gas pressure in the water as compared to that in the atmosphere. This device does not differentiate between nitrogen pressure and oxygen pressure. However, it can be used in conjunction with the Winkler method for dissolved oxygen. The saturometer determines total gas pressure, and by subtraction of oxygen, nitrogen pressures can be obtained.

FIELD WORK TO DETERMINE EXTENT AND MAJOR PROBLEMS

Intensive field work on the Columbia River was begun in 1966 to determine causes of supersaturation and its effect on salmon and steelhead trout (Ebel 1969). These studies showed that the primary cause of supersaturation was the spilling of water at dams on the river with a direct correlation between amount of spill and saturation levels. As the water spilled over the dams it entrained air, carried it to great depths where it was held under pressure and dissolved into the water. This water, upon return to lesser depths and pressure, became supersaturated with both oxygen and nitrogen.

To illustrate the effect of a change in pressure, at 10 C (50 F) one liter of air-saturated water will hold 36.25 cc of air at a depth of 6.1 m (20 ft) of water exerting a total pressure of 1208 mm of mercury. It the water is returned to the surface with a pressure of only 760 mm of mercury pressure, one liter of air-saturated water will hold only 22.8 cc of air. Thus, the water will be supersaturated to 159%.

Since passage of water through the turbines did not cause supersaturation at the dams on the Columbia and Snake rivers, there was an initial tendency on the part of researchers to ignore turbines from other sites as possible sources of supersaturation. However, studies conducted by Western Fish Toxicology Station and Bureau of Reclamation personnel in Colorado (Garton et al. 1973) found supersaturation as high as 130% produced in the turbine structures at Morrow Point Dam on the Gunnison River. This supersaturation was the result of insertion of air into the penstock to cushion the fall of water against the turbine blades. The air dissolved into the water in the deep draft tube between turbine blades and the outlet to the river. This same situation was found by MacDonald and Hyatt (1973) on the Mactaquac River in New Brunswick.

Thermal power plants were also identified as sources of supersaturation; water at a low temperature can hold more air than water at a higher temperature and when heated in a thermal power plant it becomes supersaturated. Depending upon the temperature, water will increase in saturation from 2.0 to 2.8% for every 1 C rise in temperature.

To illustrate the effect of temperature increase, at 3 C (37.4 F) and at 760 mm of mercury pressure, one liter of air-saturated water will hold 26.9 cc of air. In a power plant with a 16 C (28.8 F) temperature rise (ΔT), the effluent temperature would be 19 C (66.2 F). At this effluent temperature, a liter of air-saturated water would hold only 19.02 cc of air. If air is not released back to the atmosphere to compensate, the water will be supersaturated to 142%. A ΔT of 16 C is high for a power plant but not unreasonable and, of course, lower ΔT's also cause supersaturation but in correspondingly lesser amounts.

At the Pilgrim Plant in Massachusetts, thousands of menhaden were killed by supersaturation when they chose, because of temperature preference, the warmed but supersaturated discharge canal (Marcello et al. 1975). Since that time additional sources of supersaturation from thermal power plants have been identified in both salt and fresh water sites, such as the Green River in Wyoming (Roy Hamilton, personal communication).

Along with the question of source of supersaturation also comes the question of determining persistence of the condition. Persistence is largely determined by the ratio of surface area to volume of the body of water and by the amount of turbulence at the air-water interface. The Snake and Columbia rivers, which are deep, slow-moving river-reservoir systems, do not easily lose dissolved gases. The supersaturated condition may, at times, persist from upstream dams in Idaho and central Washington all the way to the Pacific Ocean (Ebel 1969), making supersaturation an especially serious problem. Garton et al. (1973) found that supersaturation in the Gunnison and Frying Pan rivers in Colorado was not nearly so persistent. A saturation level of 130% in the turbulent Gunnison River was reduced to 100% in less than eight miles of flow. The small turbulent Frying Pan River reduced supersaturation levels from over 115% to 100% in less than three miles of flow. May and Huston (1975) in Montana found that supersaturation in the Kootenai River (average peak flows of 65,000 cfs) persisted for 30 miles downstream. High flows (above 20,000 cfs) kept gas concentrations above 125% saturation as far as 20 miles downstream from the dam, and at times gas levels had not reached equilibrium 100 miles downstream. The Columbia River below Bonneville Dam at times remained supersaturated above 110% all the way to Astoria; while the Snake River, below Hells Canyon Dam was still supersaturated at the mouth of the Salmon River.

Measurement of effects of supersaturation on fish in the field is often difficult because of the problems of sampling for dead or injured fish in the large bodies of water affected. However, Ebel et al. (1975) and others (Weitkamp and Katz 1975) noted obvious effects on returning fishes at the ladders on the Snake and Columbia rivers. Gas bubbles were observed in both adult and juvenile fish, and mark and recapture studies demonstrated high mortality in downstream migrants. In addition, high supersaturation levels have reduced return rates of fish released for downstream migration.

Excess spilling of water over the newly-completed dam on the Kootenai River near Libby, Montana, supersaturated the water with air and killed many fish in the river downstream (May and Huston 1975). There was a marked reduction of whitefish numbers, in part from mortality of juveniles due to gas bubble disease. Large-scale suckers in the first 10-15 miles below the dam had a high incidence of gas bubble disease, but their numbers remained high, indicating that they were able to tolerate high gas concentrations, possibly, in part, due to their preference for deeper water. Complete mortality of cutthroat trout and mountain whitefish occurred in 1-5 to 3-8 days when held within two feet of the water surface at total gas levels of 131-139% saturation. In volition cages extending to 10 ft the trout still suffered 55% mortality and the whitefish suffered 67% mortality after 24 days.

"TOXICITY" STUDIES TO DETERMINE EFFECTS AND SAFE LEVELS

Although supersaturation is not a toxic substance in the classic sense, traditional toxicity studies (bioassays) were used to determine effects of supersaturation and safe levels for aquatic organisms. Additional experiments were designed along with the toxicity tests to study effects such as acclimation, avoidance, and recovery from supersaturation.

Before supersaturation could be studied in the laboratory a system had to be developed to produce supersaturated water in the test tanks. Such systems were developed simultaneously at the Western Fish Toxicology Station (Bouck et al. 1976; Nebeker et al. 1976) and by the National Marine Fisheries Service (Dawley et al. 1976). These systems attempted to simulate pressure change much like the source of supersaturation at dams. Here atmospheric air of bottled gases such as nitrogen, oxygen, or carbon dioxide were injected into water under pressure where they were dissolved to saturation at high pressures. When the pressure was relieved by release of the water into the test aquaria or tanks the water became supersaturated with the gas.

The first approach was to determine classic TL50 data for salmonid fishes of all ages including eggs, embryos, young fish through smolt stage, and the adult returning to spawn. These studies were conducted in shallow tanks (10-60 cm in depth) of varying sizes, with precise temperature control, where the fish could be exposed to carefully monitored levels of supersaturation and closely observed for effect. Both short-term and long-term chronic studies were conducted. Longer-term experiments also made possible detailed pathology studies (Figures 1, 2, 3) to determine and document the development of the "gas bubble disease" in the fish (Stroud et al. 1975). Salmonid eggs are seldom exposed to supersaturated conditions in the field. Because of the importance of supersaturation to hatcheries in the Pacific Northwest, especially where the water is heated to speed up growth of young fish, egg through swim-up studies were conducted at the Western Fish Toxicology Station. Nebeker et al. (1977a) determined that 125% supersaturation was a safe level for salmonid eggs and young sac-fry larvae in shallow tanks in the laboratory; however, when the swim-up stage developed they died at gas levels as low as 113% saturation (Figure 4). Studies conducted by Lorz and McPherson (1976) suggested that the ability of smolts to migrate and adapt to sea water may be especially sensitive to some pollutants. Nebeker et al. (1977c) conducted similar smolt studies using supersaturation as a toxicant and found that sublethal levels of supersaturation which caused gas bubble disease had no discernible effect on ability of salmonids to smolt and to acclimate to salt water.

Because salmonid stocks could be affected by the effects of supersaturation on their predators, competitors, and food organisms, other tests in addition to TL50 studies were conducted. Studies conducted at the Western Fish Toxicology Station (Bouck et al. 1976) with a predator, the largemouth bass, and juvenile salmonids in the same tank of supersaturated water demonstrated that bass could tolerate supersaturation levels that killed or injured young salmonids. Similar studies conducted with squawfish by Meekin and Turner (1974) showed that squawfish were more tolerant than salmonids but ceased to feed at higher saturation levels. Food organism studies with *Daphnia*, crayfish, and aquatic insects were conducted at the Western Fish Toxicology Station utilizing acute, long-term, and full-life cycle studies (Nebeker 1976). These studies showed that, in general, invertebrates (with the possible exception of *Daphnia magna*) were more tolerant to supersaturated water than fishes.

Because of the special nature of supersaturation, use of the classic TL50 experiments alone did not provide sufficient data to propose safe levels for aquatic life. For any given level of gas in the water the percent saturation is dependent upon both the pressure and the temperature of the body of water. A rise in water temperature or a decrease in pressure increases supersaturation. The pressure phenomenon is especially important in rivers such as the Snake or Columbia because water which is saturated to 130% at the surface, for example, will be saturated to only 100% at a depth of 10 ft. Fish staying in deeper water escape effects of supersaturation. Dawley et al. (1976), on a grant from the Environmental Protection Agency, tested juvenile chinook and steelhead trout in both shallow and deep tanks and found that fish tended to move to the slightly deeper levels with increased levels of supersaturation. Similar results were obtained by Blahm et al. (1976). However, both studies left a serious question unanswered. It was not known whether the fish stayed in the deeper areas of the tank to escape supersaturation or because the configuration of the tank made them prefer the security of the deeper water during increased stress. M. D. Knittel and coworkers at the Western Fish Toxicology Station (unpublished

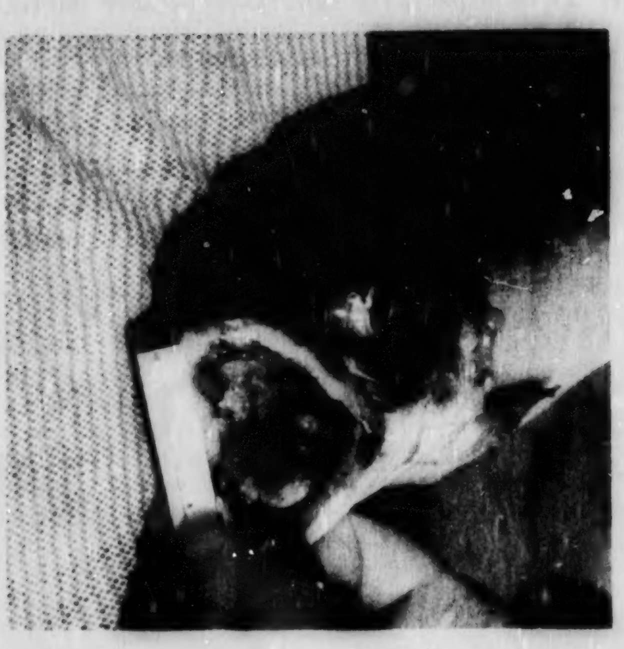

Figure 1. Adult sockeye salmon showing gas blisters in the mouth and on the left opercle.

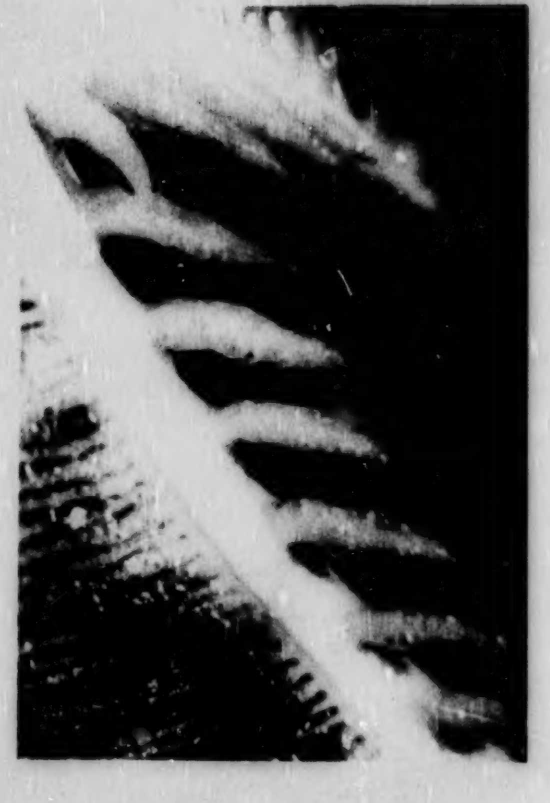

Figure 2. Gill arch of adult chinook salmon showing gas blisters on the gills and rakers.

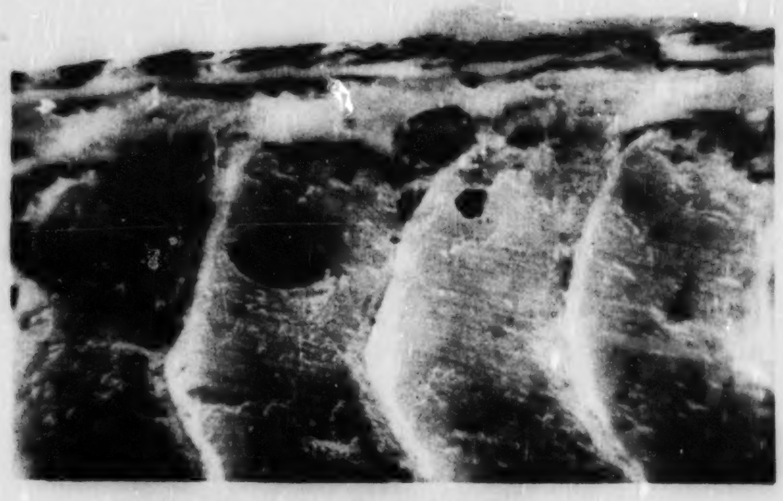

Figure 3. Cross section of adult chinook salmon flesh showing cavities (swiss cheese effect) in the muscle.

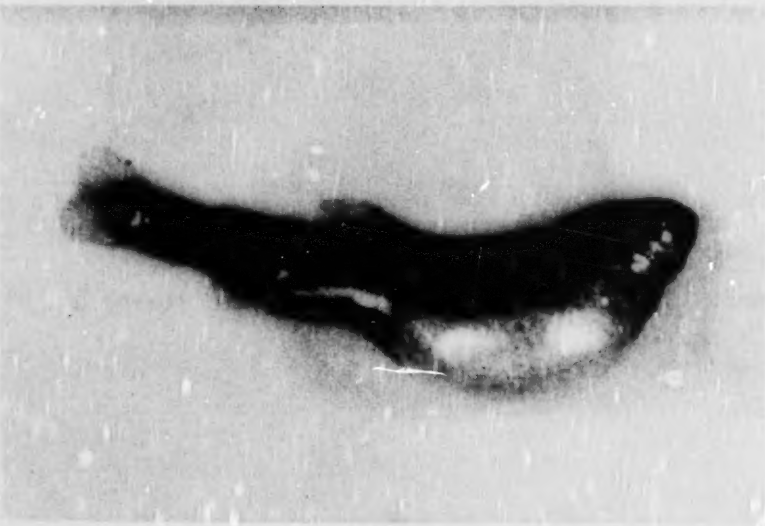

Figure 4. Steelhead trout fingerling with gas bubble disease.

manuscript) held fish in cages (30 inches in diameter by 8 inches deep) at different levels in 10 ft deep tanks. They determined that fish are able to compensate for supersaturation by moving to deeper levels in the water but that the compensation by the fish is not quite as great as one would predict. From the gas laws we assumed compensation of 5% per 20 inches increase in depth. However, they found that the compensation was only 3.7% per 20 inches increase in depth. They also found that the longevity of fish was increased by submersion to greater depths after exposure to lethal levels of gas supersaturation.

Even though it was determined that fish compensated for supersaturation by seeking greater depths there was still the question as to whether fish can sense supersaturation and whether the fish will voluntarily go to a level where supersaturation is lower. Stevens et al. (1977 unpublished manuscript) in their avoidance studies showed that the ability to detect and avoid supersaturated water seems to vary among species. Salmon were able to differentiate between different percentages of supersaturation in a pie-shaped avoidance chamber. However, trout were not clearly able to do so consistently. Avoidance of supersaturation might be secondary to behavior such as increased activity due to aggression and territoriality, a need for cover and lower light intensities, or choice of a particular current velocity. Schiewe and Weber (1976) found that bubbles in the lateral line of fish exposed to supersaturated water diminished or completely blocked the ability of the sensory units to respond to stimuli. The loss of ability to respond to stimuli decreases the fish's capability to detect objects or locate predators. Chapman and Nebeker (1977 unpublished manuscript) considered the possibility of synergism between supersaturation and the heavy metals copper and zinc, but were unable to detect any such effect. Nebeker et al. (1977b) detected effects of temperature on fish survival in supersaturated water. With juvenile steelhead trout, tested from 9 to 18 C at 116% saturation, each one degree (C) increase decreased time to 50% death by about 30 hours, from 330 hours at 9 C to about 50 hours at 18 C. Increased temperatures significantly decreased survival time of steelhead and chinook salmon, but no significant effect was apparent for sockeye or coho salmon.

One of the results of the toxicity studies was the determination of total-air-saturation water quality criteria for the protection of fish and other aquatic life. These criteria were published in the 1976 document, "Quality Criteria for Water," published by the Environmental Protection Agency in compliance with Public Law 92-500. In this document, 110% total-air-saturation was stated as a safe level for salmonid fishes in shallow water. These criteria were established with full knowledge that fish would be able to tolerate higher levels of supersaturation at greater depths. However, sublethal effects of supersaturation were noted at 110%, and lethal levels are found to be not far above 110% (Nebeker and Brett 1976). Thus, it was determined that the safe level is near 110%, especially in shallow waters of hatcheries or fish-rearing areas where depth compensation is not possible, but that deviations from this may be justified in some specific cases (Table 1, Figure 5).

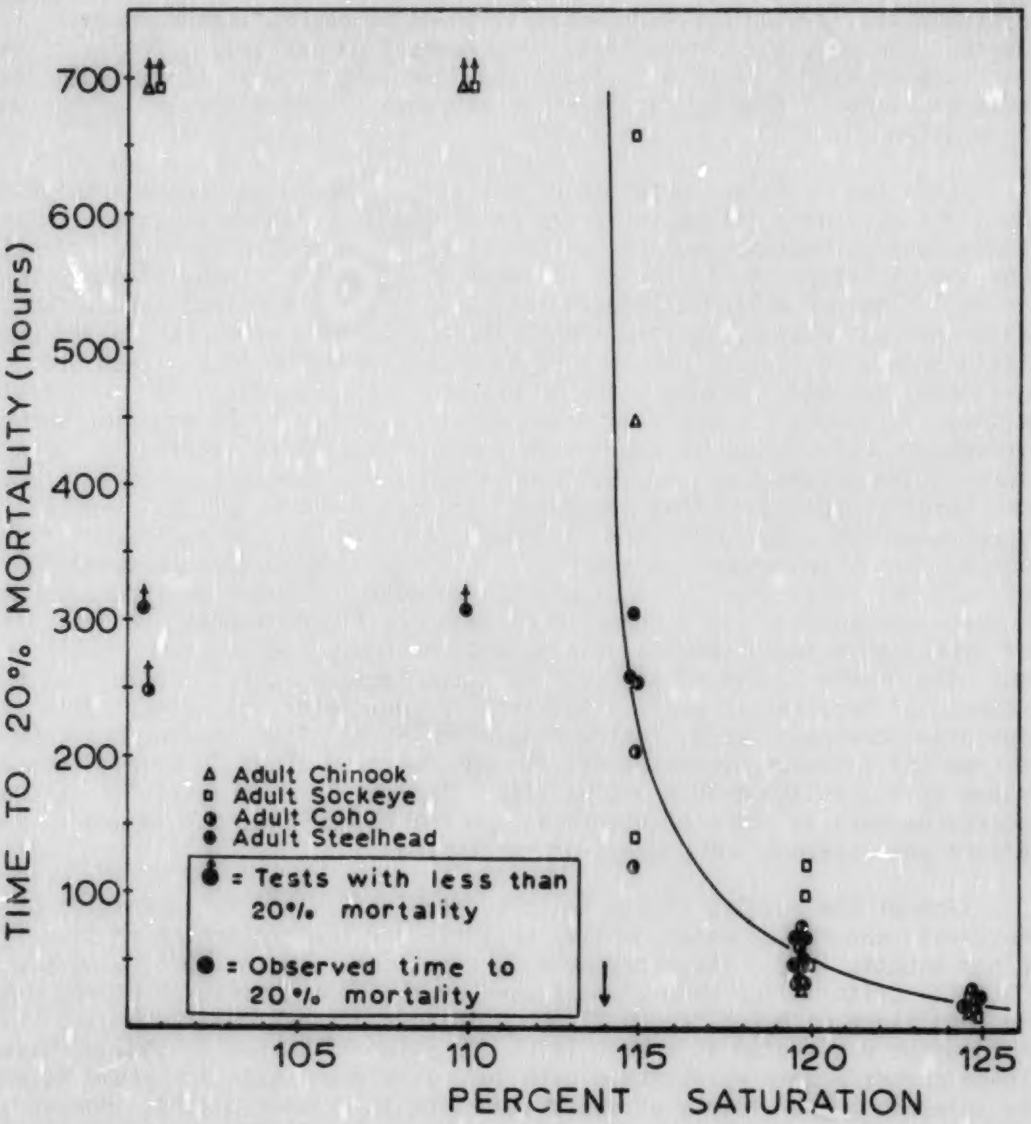

Figure 5. Determination of threshold concentration (114%) for adult salmonids (● = 10 fish/test).

TABLE 1. COMPARATIVE SENSITIVITY OF JUVENILE AND ADULT SALMONIDS AND BASS TO AIR-SUPERSATURATED WATER.

Fish	Threshold (% sat.)*
Sockeye smolts	113.6 %
Juvenile steelhead	113.8
Juvenile sockeye	114.0
Adult sockeye	114.2
Steelhead smolts	114.2
Adult coho	114.4
Adult steelhead	114.6
Adult chinook	114.7
Coho smolts	114.8
Juvenile coho	118.0
Adult bass	126.8
Juvenile bass	128.0

*based on time to 20% mortality (as determined using methods shown in Fig. 1)

SOLUTIONS

Solutions to the problem in the Pacific Northwest, in the form of structural modifications of the dams, were determined and are being implemented primarily by the Corps of Engineers. National Marine Fisheries Service personnel, funded by the Corps of Engineers, also constructed screening structures for trapping and hauling downstream migrants around problem areas. Solutions to the problem can be based either on initial planning to avoid causing supersaturation or by reduction of supersaturation when it cannot be avoided due to design of existing facilities. Reduction or elimination of supersaturation is sometimes a feasible alternative at hatcheries or other areas where a flow of waters is involved. However, reduction of supersaturation in rivers such as the Columbia is an almost-impossible task and the problem should be attacked at the start, if possible, by avoidance of supersaturation production.

Prevention of supersaturation in the Snake and Columbia river system is approached in two different ways. The first is by manipulation of river flow to avoid spilling at dams where supersaturation may be produced. The second is by physical changes in the structure of the dams themselves, such as the flip lip (Boyer 1974). Another method used to help the fishery resource is to avoid the supersaturated water completely by collecting fish with traveling screens at dams, such as Little Goose or Lower Granite on the Snake River, and trucking the fish in tank trucks to the Lower Columbia River below Bonneville Dam. This precaution has the advantage of avoiding a large part of the supersaturated river for downstream migrant fishes; however, it has the disadvantage of high trucking costs. This solution is currently under study by the Corps of Engineers and by the National Marine Fisheries Service to determine feasibility and effect of trucking, or air freighting young fish downstream. Hopefully, continuation of the field studies on young fishes will determine whether the trucking, the flow manipulations, or the physical changes are actually doing the job in reducing supersaturation effect on migrating salmonids in the Columbia-Snake river system.

SUMMARY

The supersaturation research project is an example of a case where bioassays (classic TL50 and specially designed depth compensation studies) were used in conjunction with other field and engineering research methods to set criteria and solve a specific problem. In this case "toxicity" studies were used as a definite part of the problem-solving technique. Similar studies have also been conducted on polychlorinated biphenyls, mirex, and other pollutants. This approach is likely to continue in the future when a pollutant becomes known as important, and an all-out effort is mobilized to solve the problem. Solution of the problem depends upon identifying the source and effects of the pollutant, and determining safe levels through laboratory studies. The supersaturation study was different from many in that it has not resulted in enforcement proceedings. In this case it was a cooperative effort coordinated by the Nitrogen Task Force between state pollution control agencies and the U. S. Environmental Protection Agency, and between the fishery resource agencies of the states of Oregon, Washington, and Idaho, and the National Marine Fisheries Service. The Corps of Engineers, the Bureau of Reclamation, and the Public Utility Districts shared responsibility for dam modification and other methods used to decrease the problem or its effect. In this case there was a cooperative effort to cure mistakes in design which were carried over from the past when the problem of supersaturation was well understood.

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EFFECTS OF KEPONE® ON ESTUARINE ORGANISMS[1]

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ABSTRACT

Laboratory toxicity tests were conducted to determine the effects and accumulations of Kepone in estuarine algae, mollusks, crustaceans, and fishes. Nominal Kepone concentrations calculated to decrease algal growth by 50% in static bioassays lasting seven days were: 350 µg/ℓ, *Chlorococcum* sp.; 580 µg/ℓ, *Dunaliella tertiolecta*; 600 µg/ℓ, *Nitzschia* sp.; and 600 µg/ℓ, *Thalassiosira pseudonana*. Measured Kepone concentrations calculated to cause 50% mortality in flowing-seawater toxicity tests lasting 96 hours were: 10 µg/ℓ for the mysid shrimp (*Mysidopsis bahia*); 120 µg/ℓ for the grass shrimp (*Palaemonetes pugio*); >210 µg/ℓ for the blue crab (*Callinectes sapidus*); 70 µg/ℓ for the sheepshead minnow (*Cyprinodon variegatus*); and 6.6 µg/ℓ for the spot (*Leiostomus xanthurus*). Bioconcentration factors (concentration in whole animals divided by concentration measured in water) in these tests were greatest for fishes (950 to 1,900) and less for grass shrimp (420 to 930).

Survival, growth, and reproduction of mysids and sheepshead minnows were decreased in chronic bioassays lasting 14 to 64 days. Growth of mysids and sheepshead minnows was reduced by exposure to 0.07 µg/ℓ and 0.08 µg/ℓ respectively. Bioconcentration factors for sheepshead minnows in the chronic bioassay averaged 5,200

®Registered trademark, Allied Chemical Corp., 40 Rector St., New York, 10006. Kepone was purchased from Chem Service, West Chester, PA, as 99% pure. Our analyses indicated 88% purity.

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(range, 3,100-7,000) for adults exposed for 28 days and 7,200 (3,600-20,000) for juveniles exposed for 36 days. The chronic toxicity and bioconcentration potential of Kepone are more important factors than its acute toxicity in laboratory evaluations of environmental hazard. Therefore, these factors should be considered when attempting to assess present impacts and to limit future impacts of this insecticide on the aquatic environment.

INTRODUCTION

Kepone (decachlorooctahydro-1,3,4-metheno-2H-cylobuta [cd] pentalene 2-one) is an insecticide that was manufactured and formulated in the United States to control ants, cockroaches, and insect pests of potatoes and bananas. Kepone is toxic to birds and mammals, including man (Jaeger 1976), and acutely toxic to some estuarine organisms (Butler 1963). Recent contamination of water, sediment, and biota in freshwater and estuarine portions of the James River, Virginia, has stimulated concern about this chemical's hazard to aquatic biota (Hansen et al. 1976) This concern was based on (1) the continued occurrence of Kepone in many finfishes and shellfishes in amounts that forced closure of fishing because of potential human health hazard, and (2) laboratory studies which showed that Kepone is highly bioaccumulative and toxic to estuarine organisms, particularly in chronic exposures. This paper describes the results of these laboratory toxicity tests with estuarine algae, oysters, crustaceans, and fishes and chronic tests with a crustacean and a fish.

EXPERIMENTAL PROCEDURES

Acute Toxicity

Algae: The unicellular algae *Chlorococcum* sp., *Dunaliella tertiolecta*, *Nitzschia* sp., and *Thalassiosira pseudonana* were exposed to Kepone for seven days to determine its effect on growth (Walsh et al. 1977). Algae were cultured in 25 or 50 ml of growth media and artificial seawater of 30 $^0/oo$ salinity and a temperature of 20 C (Hollister et al. 1975). Kepone, in 0.1 ml acetone, was added to culture media, and 0.1 ml of acetone was added to control cultures. Photoperiod consisted of 12 hours dark and 12 hours of 5000 lux illumination. Effect on growth was determined by electrophotometrically measuring optical density. Also, algae grown for 6 days in media and then exposed to 100 μg/ℓ Kepone for 24 hours were analyzed for Kepone content.

Oysters: The acute toxicity of Kepone to embryos of the eastern oyster (*Crassostrea virginica*) was determined by measuring its effect on development of fully-shelled, straight-hinged veligers in a 48-hour static exposure[1]. Methods used were those of Woelke (1972) and U. S. EPA (1975). Test containers were 1-ℓ glass jars that contained 900 mℓ of 20 C, 20 ^{o}/oo salinity seawater and 25,000 ± 1,000 oyster embryos. All test concentrations were triplicated. The number of normal and abnormal embryos were counted microscopically in a Sedgewick-Rafter cell at the end of 48 hours of exposure to Kepone.

Crustaceans and Fishes: The acute toxicity of Kepone to grass shrimp (*Palaemonetes pugio*), blue crabs (*Callinectes sapidus*), sheepshead minnows (*Cyprinodon variegatus*), and spot (*Leiostomus xanthurus*) was determined in 96-hour flow-through toxicity tests (Schimmel and Wilson 1977). Acclimation and testing procedures were compatible with those of Standard Methods (APHA 1971). Test animals were caught locally and 20 were placed in each 18ℓ aquarium. Water flow to each aquarium was 68 ℓ/hour. Stock solutions of Kepone in acetone were metered into experimental aquaria at the rate of 60 mℓ/hour. Control aquaria received 60 mℓ of acetone/hour. At the end of the experiment, surviving animals were chemically analyzed for Kepone content.

The acute toxicity of Kepone to mysids (*Mysidopsis bahia*) was determined by using intermittent flows of water from a diluter (Mount and Brungs 1967) or continuous flow of water from a siphon and Kepone from an infusion pump (Bahner et al. 1975). Thirty-two 48-hour-old juvenile mysids were placed in chambers (4 mysids per chamber) in each test aquarium. Chambers consisted of glass petri dishes to which a 15 cm tall cylinder of 210μ mesh nylon screen was glued. Water in the chambers was renewed by a self-starting siphon which nearly emptied and then filled each aquarium at about 25 min intervals.

Chronic Toxicity

Mysidopsis bahia: The chronic toxicity of Kepone to this mysid was determined in 19-day exposures that began with 48-hour-old juveniles. (Nimmo et al., in press). The time permitted production of several broods for assessment of reproductive success and survival of progeny. Exposure conditions, apparatus, and number of mysids per concentration were identical to those of the acute toxicity tests. Three tests were conducted: One to assess effects on survival and reproduction, and two at lower concentrations to determine effects on growth. Data from the two growth experiments were pooled for statistical analysis.

Cyprinodon variegatus: The chronic toxicity of Kepone to sheepshead minnows was determined in a 64-day flow-through bioassay--exposure of adults for 28 days followed by a 36-day exposure of their progeny (Hansen et al. 1977). We delivered Kepone, 0.0088 μℓ of the solvent triethylene glycol, and 1.5ℓ of filtered 30 C seawater (average salinity, 15 ^{o}/oo; range, 8-26 ^{o}/oo) to each 70ℓ aquarium during each of 440 daily cycles of the dosing apparatus of Schimmel et al. (1974). Seawater and solvent were delivered to the control aquarium. Thirty-two adult females and 32 adult males were exposed to each concentration of Kepone for 28 days. Egg production was enhanced using injections of 50 I.U. of human chorionic gonadotrophic hormone on exposure day 25 and 27 (Schimmel et al. 1974). Eggs were fertilized on day 28 and placed in chambers (glass petri dishes with 9-cm tall cylinders of 450μ nylon mesh). Twenty embryos were used in each chamber. Embryos from control fish were placed in four chambers in the control aquaria and in four chambers in each of the six aquaria receiving Kepone. Embryos from fish in each of the six aquaria receiving Kepone were placed in four chambers in that aquarium and in four chambers in the control aquarium. Water in the chambers was exchanged by the action of a self-starting siphon in each aquarium that caused water levels to fluctuate 5 cm about 40 times per day. In the 36-day exposure to determine Kepone's effect on survival and growth of progeny, embryos hatched and fry grew until they were juvenile fish. Kepone content of adult fish, their eggs, and juvenile fish was determined.

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[1] This research was performed under an EPA contract by Tom Heitmuller, Bionomics-EG&G, Inc. Marine Research Laboratory, Pensacola, Florida 32507.

STATISTICAL ANALYSES

Probit analyses of growth and mortality data were used to determine EC50's and LC50's. Growth data for *M. bahia* were subjected to analysis of variance ($\alpha = 0.05$) and for *C. variegatus*, analysis of covariance and Newman-Kuels tests ($\alpha = 0.01$) was used.

CHEMICAL ANALYSES

Water from acute and chronic tests with crustaceans and fishes, and organisms surviving these tests, were analyzed by gas chromatography. Methods of extraction concentration, cleanup, and quantification were described by Schimmel and Wilson (1977).

RESULTS AND DISCUSSION

Acute Toxicity

Algae: Growth of marine unicellular algae was reduced by exposure to Kepone in static tests (Table 1). *Chlorococcum* was the most sensitive of the four algae tested with a 7-day EC50 of 350 μg/ℓ. The three less sensitive species responded similarly to Kepone with overlapping confidence limits for EC50's. Algae exposed to 100 μg Kepone/ℓ of media accumulated the chemical with *Chlorococcum* containing 0.80 μg/g; *D. tertiolecta*, 0.23 μg/g; *Nitzschia*, 0.41 μg/g; and *T. pseudonana*, 0.52 μg/g. Butler (1963) reported that when estuarine phytoplankton were exposed to 1,000 μg/ℓ carbon fixation was reduced by 95%.

Oysters: The 48-hr EC50 for oyster larvae in static tests was less than those of algae (Table 1). The EC50, calculated using nominal water concentrations, was 66 μg/ℓ[2]. Embryos from 56 μg/ℓ were fully shelled and straight-hinged but appeared smaller than those from controls. The percentage of normal embryos in 65 μg/ℓ was 32 percent and in 87 μg/ℓ it was 0%. The concentration of Kepone calculated to reduce shell deposition of juvenile eastern oysters by 50% in a 96-hour flowing water bioassay was 38 μg/ℓ in water of 14 C and 11 μg/ℓ in water of 31 C (Butler 1963).

TABLE 1. ACUTE TOXICITY OF KEPONE TO ESTUARINE ORGANISMS. ALGAL AND MOLLUSK TOXICITY TESTS WERE STATIC AND ESTIMATED NOMINAL CONCENTRATIONS REDUCING GROWTH OF ALGAE AND EMBRYONIC DEVELOPMENT OF OYSTERS BY 50% (EC50). TOXICITY TESTS WITH CRUSTACEANS AND FISHES WERE FLOW-THROUGHS THAT ESTIMATED THE MEASURED CONCENTRATION IN WATER LETHAL TO 50% (LC50). NINETY-FIVE % CONFIDENCE LIMITS ARE IN PARENTHESES.

Organisms	Temperature, C	Salinity, °/oo	Exposure Duration, Days	EC50/LC50 µg/ℓ	
Algae					
Chlorococcum sp.	20	30	7	350	(270-400)
Dunaliella tertiolecta	20	30	7	580	(510-640)
Nitzschia sp.	20	30	7	600	(530-660)
Thalassiosira pseudonana	20	30	7	600	(500-700)
Mollusk					
Crassostrea virginica	20	21	2	66	(60-74)
Crustaceans					
Callinectes sapidus	19	20	4	>210	
Mysidopsis bahia	26	13	4	10	(8.1-12)
Palaemonetes pugio	20	16	4	120	(100-170)
Fishes					
Cyprinodon variegatus	18	15	4	70	(56-99)
Leiostomus xanthurus	25	18	4	6.6	(5.3-8.8)

Crustaceans and Fishes: Kepone, at the concentrations tested, was acutely toxic to mysids (Nimmo et al. 1977), grass shrimp, sheepshead minnows, and spot, but not to blue crabs (Schimmel and Wilson 1977) (Table 1). Spot and mysids were the more sensitive species with 96-hour LC50 values of 6.6 and 10 µg/ℓ. Crabs exposed to as much as 210 µg Kepone/ℓ suffered no significant mortality. Symptoms of acute Kepone poisoning in fishes included lethargy, loss of equilibrium, and darkened coloration on the posterior portion of the body, occasionally only in one quadrant. Crustaceans became lethargic before death but exhibited no color change. Butler (1963) reported 48-hour LC50 or EC50 values (based on nominal concentrations) for other estuarine organisms were: brown shrimp (*Penaeus aztecus*), 85 µg/ℓ; and white mullet (*Mugil curema*), 55 µg/ℓ.

Kepone was bioconcentrated from water by all four species we exposed for 96 hours. Bioconcentration factors (concentration in tissue divided by measured Kepone in water) for fishes were similar (950 to 1,900). Bioconcentration factors for grass shrimp ranged from 420 to 930 and for blue crabs, 6 to 10.

CHRONIC TOXICITY

Mysidopsis bahia: Exposure of this mysid to Kepone for 19 days in the first experiment decreased its survival and reduced the number of young produced per female (Table 2) (Nimmo et al. 1977). At the highest concentration (8.7 μg/ℓ) all mysids were dead within the first two days. At lesser concentrations (1.6 and 4.4 μg/ℓ) mortality continued throughout the test. Eighty-four % of the mysids survived exposure to 0.39 μg Kepone/ℓ water and 91% survived in control aquaria. In addition, natural reproduction was affected. Average number of young mysids produced per female was 15 in control, 9 in 0.39 μg/ℓ, and 0 in 1.6 μg/ℓ. Mysids that survived throughout the Kepone exposure appeared smaller than those in control aquaria, therefore, two additional experiments were conducted to measure Kepone's effect on growth.

TABLE 2. EFFECT OF KEPONE ON THE SURVIVAL OF *MYSIDOPSIS BAHIA* AND ON AVERAGE NUMBER OF YOUNG PER FEMALE IN A 19-DAY FLOW-THROUGH TOXICITY TEST.

Average Measured Kepone Concentration (μg/ℓ)	Percentage Survival	Number of Young per Female
Control	91	15.3
0.39	84	8.9*
1.6	50	0
4.4	3	--
8.7	0	--

*Statistically significant at $\alpha = 0.05$ using 2 sample t-test.

In these experiments, the average length (tip of carapace to end of uropod) of mysids exposed to Kepone was decreased (Nimmo et al. 1977). Females exposed to 0.072, 0.11, 0.23, or 0.41 μg/ℓ were significantly shorter than were control mysids; average length was 8.2 mm for exposed versus 8.6 mm for control female mysids. Unexposed and exposed males, however, were of similar average lengths, 7.7 to 8.0 mm.

Cyprinodon variegatus: Kepone was toxic to adult sheepshead minnows exposed for 28 days (Table 3). Symptoms of poisoning included: scoliosis, darkening of the body posterior to the dorsal fin, hemorrhaging near the brain, edema, fin-rot, uncoordinated swimming, and cessation of feeding. Symptoms were first observed on day 1 in 24 μg/ℓ, 2 in 7.8 μg/ℓ, 3 in 1.9 μg/ℓ, and day 11 in 0.8 μg/ℓ. Mortalities began 5 to 8 days after onset of symptoms.

TABLE 3. EFFECT OF KEPONE ON AND ACCUMULATION OF KEPONE BY ADULT SHEEPSHEAD MINNOWS EXPOSED FOR 28 DAYS.

Average Measured Exposure Concentration, μg/ℓ	Percentage Mortality	Whole Body Concentration, μg/g
ND*	5	ND
0.05	5	0.30
0.16	0	0.78
0.80	22	3.0
1.9	80	12.
7.8	100	
24.	100	

*ND = Kepone not detected in control water (<0.02 μg/ℓ) nor in control fish (<0.02 μg/g).

Kepone affected the progeny of 28 day exposed adults. In Kepone-free water, mortality of embryos from adults exposed to 0.05-0.8 μg/ℓ was similar to that of embryos from unexposed adults (range, 6-12 percent). However, in Kepone-free seawater, 25% of the embryos from fish exposed to 1.9 μg of Kepone/ℓ died; abnormal development of 13 of these 20 embryos preceded mortality.

Kepone in water affected progeny of exposed parents to a greater extent than progeny of unexposed parents (Table 4). Some embryos exposed to 2.0 μg/ℓ developed abnormally and fry had more pronounced symptoms and they began to die 10 days earlier when parental fish had been exposed to 1.9 μg/ℓ than was observed in progeny from unexposed parents.

Kepone also affected growth of sheepshead minnows in the 36-day exposure of progeny (Figure 1). The average standard length of juveniles exposed to all Kepone concentrations was less than that of unexposed control juveniles. Lengths decreased in direct proportion to increasing Kepone concentrations in water and were generally not influenced by parental exposure. A similar decrease was also noted in weights, but because juveniles exposed to 0.72, 2.0, or 6.6 μg/ℓ were edematous, they weighed more than unexposed juveniles of similar lengths.

Kepone was bioconcentrated by sheepshead minnow adults and their progeny exposed to the insecticide in water. Kepone was bioconcentrated in adult fish in direct proportion to concentration in exposure water (Table 3). Concentration factors averaged 5,200 (range, 3,100-7,000). Kepone concentrations in females and their eggs were similar and were 1.3 times greater than amounts in males. Concentrations of Kepone in juvenile fish, at the end of the 36-day progeny exposure, increased with increased concentration of Kepone in water (Table 4). Prior exposure of parental fish apparently did not affect final Kepone concentration in progeny. Concentration factors for juvenile fish averaged 7,200 (range, 3,600-20,000) and increased with decrease in concentration of exposure.

TABLE 4. MORTALITY IN PROGENY OF ADULT SHEEPSHEAD MINNOWS THAT WERE EXPOSED TO KEPONE AND IN PROGENY OF UNEXPOSED, CONTROL FISH. NOMINAL EXPOSURE FOR THE 28-DAY EXPOSURE OF ADULT FISH AND THE 36-DAY EXPOSURE OF PROGENY WERE THE SAME. PROGENY EXPOSURE BEGAN WITH EMBRYOS AND ENDED WITH JUVENILE FISH FROM THE EMBRYOS. RESIDUES ARE CONCENTRATIONS OF KEPONE (μg/g) IN WHOLE JUVENILES, WET WEIGHT.

Measured Exposure Concentration	Parental Fish History			
	Progeny of Unexposed Parents		Progeny of Exposed Parents	
μg/ℓ	Mortality %	Residue μg/g	Mortality %	Residue μg/g
Control (ND)	10	ND[1]	10	ND[1]
0.08	22	1.1	9	1.6
0.18	12	1.4	18	1.0
0.72	28	2.6	18	1.9
2.0	40	7.8	62	8.4
6.6	40	22.	--	--
33.	100	--	--	--

[1] ND = not detectable, <0.02 μg/ℓ, <0.02 μg/g.

In our tests, Kepone was acutely toxic to, and accumulated by, estuarine algae, mollusks, crustaceans, and fishes. Chronic toxicity tests with *M. bahia* and *C. variegatus* revealed that Kepone affected survival, growth, and reproduction. Effects on growth were observed at 0.001 of the 96-hour LC50. Accumulation of Kepone was also greatest in chronic tests. Therefore, chronic tests should be used to assess Kepone's environmental hazard and to make decisions necessary to minimize its future impact on the aquatic environment.

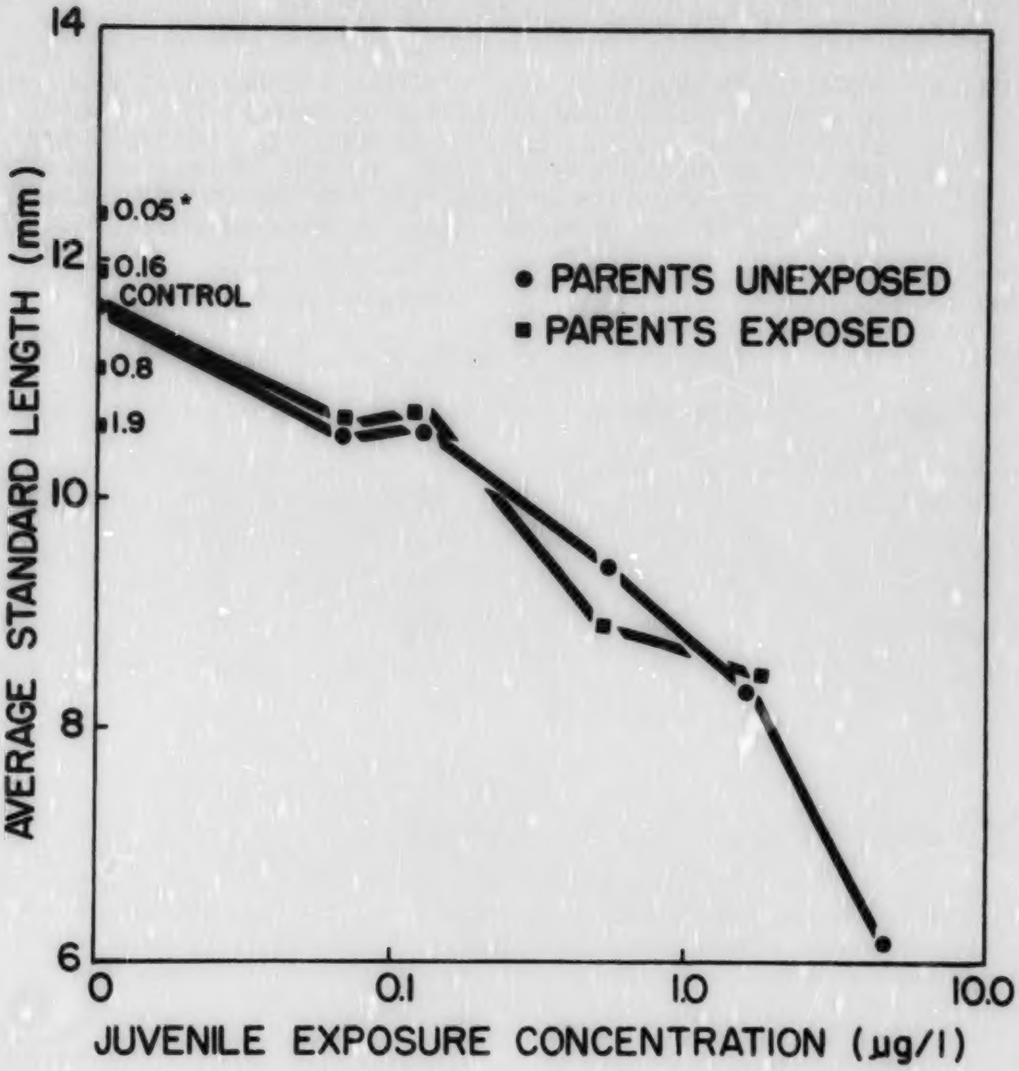

Figure 1. Average standard length of juvenile sheepshead minnows exposed as embryos, fry, and juveniles for 36 days to 0, 0.08, 0.18, 0.72, 2.0, or 6.6 µg of Kepone/ℓ of water. Parent fish in some instances also were exposed to similar concentrations of Kepone: 0, 0.05, 0.16, 0.80, or 1.9 µg/ℓ.

*Concentration of Kepone in water, µg/ℓ, for parent fish exposed prior to placement of their embryos in Kepone-free water.

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COLLAGEN METABOLISM IN FISH EXPOSED TO ORGANIC CHEMICALS

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ABSTRACT

One major function of collagen is to serve as the structural support for bones. Fish grow throughout life and the vertebrae were assumed to enlarge and elongate in proportion to growth. The synthesis of vertebral collagen and hydroloproline was examined as an indicator of growth, and as a sensitive predict of the chronic effects of toxaphene, Aroclor 1254, the dimethylamine salt of 2,4-D, and di-2-ethylhexyl phthalate. Rainbow trout (Salmo gairdneri), brook trout (Salvelinus fontinalis), fathead minnows (Pimephales promelas), and channel catfish (Ictalurus punctatus) were the species tested in chronic toxicity experiments, and collagen was reduced by all four chemicals. Interpretation of collagen synthesis data required information on vitamin C distribution in liver and bone since the vitamin is involved in the hydroxylation and detoxification of organic chemicals in liver and of collagen synthesis in bone. Toxaphene reduced the vitamin C content of vertebrae in channel catfish, but vitamin C content in the liver remained constant or showed a slight increase. The reduction of vitamin C in bone is thought to inhibit collagen formation. Within limits, collagen synthesis can be interpreted as a sensitive indicator and predictor of fish growth.

INTRODUCTION

Chronic toxicity studies of contaminant effects on fish are expensive, high-risk investigations that require from 10 months to a year to conduct. Such studies commonly include measurement of the long-term effects of a contaminant on growth, reproduction, and survival of adults, and growth and survival of the offspring. Consequently, there is much interest in developing alternative methodologies that provide similar information with less effort and expense. Grant and Schoettger (1972) stated that biochemical factors in fish that can be correlated with toxicant exposures and residues should provide a useful means of anticipating the subtle, adverse effects of organic contaminants on fish. However, investigators have used various biochemical indicators of chronic effects without establishing the significance of such indicators to growth, reproduction, and survival. Biochemical monitoring cannot rely on unsupported assumptions, since the biochemical adaptive capacity of the fish can lead to broad erroneaous conclusions.

Growth of fish is usually evaluated by measuring weight or length; however, biochemical changes due to contaminant intoxication would occur before reductions in growth are observed. Measurement of biochemical changes should therefore decrease the time required for chronic toxicity determinations. Initially, we selected vertebral collagen content and the hydroxyproline concentration in collagen as potential indicators of growth and development in fish. These biochemical characteristics were incorporated for evaluation into a general chronic toxicity study of toxaphene that was conducted to establish water quality criteria for this insecticide (Mayer et al. 1975, 1977; Mehrle and Mayer 1975). Subsequently, our evaluations of biochemical characteristics were extended to toxicological studies of Aroclor 1254 (polychlorinated biphenyl), the dimethylamine salt of 2,4-D (2,4-D DMA), and di-2-ethylhexyl phthalate (DEHP). Our results are summarized in this report.

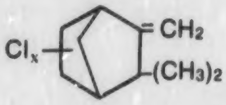
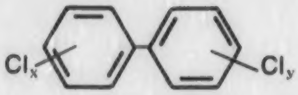
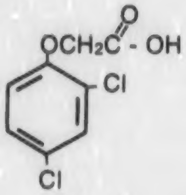
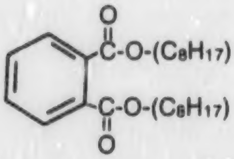
METHODS AND MATERIALS

Experimental Design

Rainbow trout (*Salmo gairdneri*), brook trout (*Salvelinus fontinalis*), fathead minnows (*Pimephales promelas*), and channel catfish (*Ictalurus punctatus*) were continuously exposed to toxaphene, Aroclor 1254, 2,4-D DMA, and DEHP in water (Table 1). The exposure systems were proportional diluters modeled after Mount and Brungs (1967) and modified as recommended by McAllister et al. (1972). Acetone was used as the carrier solvent for all chemicals except 2,4-D DMA, for which distilled water was the solvent. Flow-splitting chambers as desinged by Benoit and Puglisi (1973) were used to thoroughly mix and divide each chemical concentration for delivery to the exposure tanks. Artificial daylight was provided by the method of Drummond and Dawson (1970), and water temperatures were maintained within ± 0.2 C.

Eggs and fish were maintained as recommended by Brauhn and Schoettger (1975) before and during the studies. Studies on rainbow and brook trout and fathead minnows were conducted according to the recommended procedures for

Table 1. Chemicals tested against fish to determine their effects on the collagen and hydroxyproline concentrations in vertebrae.

Chemical	Type	Use	Structure
Toxaphene	Chlorinated Camphene	Insecticide	 Clx = CH2 (CH3)2 x=4-10 (67-69% Cl)
Aroclor 1254	Polychlorinated Biphenyl	Dielectric Fluid	 x+y = 3-8 (54% Cl)
2, 4-D DMA	Phenoxyacetic Acid	Herbicide	 OCH2C - OH O Cl Cl
Di-2-ethylhexyl Phthalate	Phthalic Acid Ester	Plasticizer	 O C-O-(C8H17) C-O-(C8H17) O

chronic tests with brook trout and fathead minnows (U.S. Environmental Protection Agency 1972 a,b). Test procedures for channel catfish were described by Mayer et al. (1977).

To determine the interactive effects of organochlorine contaminants and dietary vitamin C, we continuously exposed 10-month old channel catfish to a concentration series ranging from 37 to 475 ng/l of toxaphene. Within each concentration, the fish were subdivided into three groups, and each group was fed ad libitum a modification (Mehrle et al. 1977) of the Oregon Test Diet (National Academy of Science 1973) containing 63, 670, or 5,000 mg/kg of vitamin C. The amount recommended by the Academy is 100 mg/kg.

The designs of the experiments were completely randomized or randomized block (Cochran and Cox 1968). Growth and biochemical data were analyzed statistically by analysis of variance, and treatment means were compared by using a least significant difference test with the level of significance at $P \leq 0.05$ (Snedecor 1965). Linear regression analyses were calculated to determine the relation of vitamin C distribution in liver and vertebrae to exposure concentrations of toxaphene, and the relation of vertebral collagen and hydroxyproline to fish weight. Weight was presented as percentage of the weight of control fish for graphical simplification.

Growth Measurements and Biochemical Analyses

The fish were weighed and biochemical determinations were made at times scheduled for each study. In this paper, however, we have limited the data presented to those measurements made at the end of the exposures. (The toxaphene-vitamin C interaction study included growth and biochemical determinations made after channel catfish fingerlings had been exposed to toxaphene for 90 and 150 days.) A summary of experimental conditions is presented in Table 2. Backbones (vertebrae) were dissected from the fish and collagen, calcium, and phosphorus concentrations were determined; hydroxyproline was determined for each isolated collagen fraction. The vertebrae were dried at 110 C for 2 h in a forced-air oven, split into two fractions, and weighed. Collagen was isolated from one fraction by the method of Flanagan and Nichols (1962). The isolated collagen was weighed and subjected to hydrolysis at 115 C in 5 ml of 6 N HCl for 16 h. Hydroxyproline was determined in a 2-ml sample (Woessner 1961). The other bone fraction was subjected to hydrolysis at 115 C in 3 ml of 6 N HCl for 16 h. In this hydrolysate, calcium was determined by atomic absorption spectrophotometry and phosphorus by the Fiske and Subbarow method (1925). In very young fry, only the whole-body hydroxyproline content was analyzed. Vitamin C was determined (Hubmann et al. 1969) on each bone and liver sample in the toxaphene-vitamin C study with channel catfish fingerlings. Protein measurements were performed according to Lowry et al. (1959) on rainbow trout fry. Fathead minnows and channel catfish exposed to toxaphene were x-rayed to determine changes in vertebral structures.

Table 2. Summary of experimental conditions during continuous exposure of fish to organic chemicals.

Chemical, species, and life stage	Chemical concentration	Water temperature ($^{\circ}$C)	Age at initiation of exposure	Duration of fish exposure (days)
Toxaphene				
Brook trout				
Fry	39-502 ng/l	9	Eyed eggs[b]	90
Fathead minnow				
Fry	13-173 ng/l	25	40 days	98
Fry	94-727 ng/l	25	10 days	150
Channel catfish				
Fry	49-630 ng/l	26	0[c]	90
Fingerlings[a]	37-475 ng/l	26	10 mo	90,150
Aroclor 1254				
Brook trout				
Fry	0.43-6.2 μg/l	12	Eyed eggs[d]	118
2,4-D DMA				
Fathead minnow				
Adult	0.20-2.0 mg/l	25	9 mo	60
Di-2-ethylhexyl phthalate				
Rainbow trout				
Fry	5.0-54 μg/l	10	Eyed eggs[d]	90
Brook trout				
Adult	3.7-52 μg/l	9-15	1.5 yr	150
Fathead minnow				
Fry	11-100 μg/l	25	10 days	127

[a] Toxaphene-vitamin C interaction study.

[b] Exposed 22 days before hatching.

[c] Eggs and fry were produced by exposed parents and remained exposed.

[d] Exposed 10 days before hatching.

RESULTS AND DISCUSSION

Rationale for Monitoring Collagen

Collagen is the major fibrous protein of all vertebrates and most invertebrates (Piez and Likens 1958), and in vertebrates it functions as the organic matrix of connective tissues and bones. The collagen molecule is unique in its amino acid content (Harrington and von Hippel 1963); the amino acids hydroxyproline and proline together make up about one-tenth, and glycine one-third, of all the amino acids in collagen. Hydroxyproline is found only in two proteins--collagen and elastin. The contribution of elastin to the total hydroxyproline content is negligible, since the total amount of elastin is much smaller than that of collagen, and since the hydroxyproline content of elastin is only one-tenth that in collagen (Green et al. 1968). The synthesis of collagen, like that of other proteins, occurs on the ribosomes in fibroblasts, osteoblasts, and chondroblasts, and the hydroxylation of proline and lysine occurs after they are incorporated into the polypeptide protocollagen. The enzyme collagen hydroxylase (peptidyl proline hydroxylase) begins activity during gastrulation and catalyzes hydroxylation; vitamin C, α-ketoglutarate, and ferrous ion serve as cofactors for the enzyme (Mussini et al. 1967).

The importance of collagen in animals is shown by its wide distribution and many fucntions during growth, development, and wound healing. One function is to serve as the structural support for bones. Dried bone consists of one-third organic matrix and two-thirds minerals. About 90% of the organic matrix is collagen, and the rest consists of mucopolysaccharides, mucoproteins, and lipids (Nusgens et al. 1972). Calcification and mineralization take place around and within the collagen fibrils in bone and, as development proceeds, the deposition of calcium and phosphate produces mature bone.

The use of collagen as a representative differentiated protein in the study of embryonic development has been reported in amphibian embryological investigations (Green et al. 1968; Rollins and Flickinger 1972). Collagen synthesis, though repressed during the first embryonic cleavage stages of the frog *Xenopus laevis*, begins during gastrulation and increases 500-fold through neurulation, hatching, and posthatching stages. Decreased excretion of hydroxyproline in urine has also shown promise as an indicator of nutritional deficiency and reduced growth rates in humans (Whitehead and Coward 1969). Inasmuch as fish continue to grow throughout life, and their vertebrae continue to elongate and enlarge with growth, we hypothesized that backbone development should increase in proportion to growth, and that collagen and hydroxyproline concentrations would be indicators of this growth.

Relation of Vertebral Collagen and Hydroxyproline to Growth

We found that vertebral collagen and hydroxyproline concentrations were usually sensitive biochemical indicators of growth in fish exposed to toxaphene (Table 3). The reduction in weight caused by toxaphene in brook trout fry occurred 23 to 30 days after reductions in whole-body hydroxyproline content were observed (Mayer et al. 1975). In the first fathead minnow study

Table 3. Weight and backbone composition of fish continuously exposed to organic chemicals.

Chemical, species, and concentration	Fish weight (g)	Backbone composition		
		Collagen (mg/g)[a]	Hydroxyproline (mg/g)[b]	inorg/org constituents[c]
Toxaphene (ng/l)				
Brook trout				
0	0.81	300	19	0.70
39	0.44*	250*	16*	1.24
68	0.59	250*	16*	1.64
139	0.43*	250*	16*	1.64
Fathead minnow				
0[d]	1.28	323	31	0.49
94	1.14*	269*	24*	0.69
205	1.01*	229*	14*	1.17
399	1.14*	199*	23*	1.23
727	1.04*	224*	26*	1.24
0[e]	1.02	190	30	0.62
13	1.12	220	29	0.56
25	0.95	200	29	0.58
54	1.01	180	24*	0.70
97	0.86*	140*	24*	0.86
173	0.79*	150*	25*	0.79
Channel catfish				
0	1.56	270	58	0.48
49	1.48	260	53	0.57
72	1.48	240*	47*	0.72
129	1.50	240*	51*	0.65
299	1.00*	240*	52*	0.59
630	1.10*	230*	51*	0.59
Aroclor 1254 (µg/l)				
Brook trout				
0	0.68	454	29	0.26
0.43	0.64	437	23*	0.23
0.69	0.65	351*	21*	0.53
1.5	0.64	435	23*	0.41
3.1	0.52	386*	23*	0.60
6.2	0.74	397*	25*	0.80
2,4-D DMA (mg/l)				
Fathead minnow				
0	1.79	456	28	0.29
0.2	1.55	470	28	0.29
0.3	1.68	462	30	0.28
0.5	1.53	436	34*	0.33
1.0	1.69	410*	26	0.49
2.0	1.65	373*	34*	0.70

Table 3. (continued)

Chemical, species, and concentration	Fish weight (g)	Backbone composition Collagen (mg/g)[a]	Hydroxyproline (mg/g)[b]	Inorg/org constituents[c]
DEHP (μg/l)				
Rainbow trout				
0	0.94	175	52	-[f]
5	0.92	158	50	-
14	0.96	123*	40	-
54	1.07	144*	50	-
Brook trout				
0	453	445	37	0.49
3.7	434	378*	47*	0.56
7.9	424	391*	47*	0.60
13	428	371*	50*	0.68
23	422	388*	45*	0.64
52	419	381*	47*	0.65
Fathead minnow				
0	0.93	366	24	0.46
11	0.93	293*	30*	0.57
15	0.95	292*	30*	0.62
26	0.92	250*	35*	0.69
52	0.91	172*	26	0.92
100	0.98	171*	26	0.94

[a] Collagen in dry backbone.

[b] Hydroxyproline in dry collagen, except in toxaphene-brook trout study which was mg/g dry bone.

[c] Calcium + phosphorus ÷ collagen in dry bone.

[d] First test (Mehrle and Mayer 1975).

[e] Second test (Mayer et al. 1977).

[f] Calcium and phosphorus not analyzed.

[*] Values significantly different from the controls ($P < 0.05$).

(Mehrle and Mayer 1975), adult fish weight and vertebral concentrations of collagen and hydroxyproline were all significantly reduced at all toxaphene concentrations (94-727 ng/l). In the second fathead minnow study Mayer et al. (1977) reported that the hydroxyproline concentration in backbone collagen of adults was significantly reduced by toxaphene concentrations as low as 54 ng/l, whereas weight was significantly reduced only by concentrations of 97 and 173 ng/l; in fathead minnow offspring, however, weights were reduced by exposures to 54-173 ng/l, and this measurement was more sensitive than hydroxyproline as an indicator of toxaphene effects. Growth of channel catfish fry was not reduced by toxaphene until 30 days after the eggs hatched, but the hydroxyproline content of eggs from exposed adults was significantly reduced. The effects of toxaphene on hydroxyproline occurred in concentrations ranging from 72 to 630 ng/l, whereas effects on weight were observed only in the 299 and 630 ng/l exposures.

The correlation of vertebral collagen and hydroxyproline with fish weight was relatively high in the fish exposed to toxyphene (Fig. 1). Correlation coefficients (r) tended to be higher with collagen (r = 0.626-0.911) than with hydroxyproline (r = 0.179-0.911). The relation of vertebral collagen and hydroxyproline to growth was lowest among channel catfish, but the correlation coefficient (0.651) of whole-body hydroxyproline and weight was much higher at 15 days of age than it was at 90 days (Mayer et al. 1977). The differences observed in time may have been caused by excessive mortality. Cumulative mortality continued to increase in the 72- and 129-ng/l concentrations throughout the 90-day exposure, even though mortality was statistically significant only for fish in the 299- and 630-ng/l concentrations. The continued mortality probably negated further decreases in growth by eliminating the more susceptible fish. The effect of mortality was more evident in the study of brook trout exposed to Aroclor 1254, where no significant effects on weight were observed in fry continuously exposed for 118 days (Table 2). The correlation coefficients were low for both collagen (r = 0.183) and hydroxyproline (r = 0.333). However, weight was significantly reduced in the 1.5 to 6.2 μg/l concentrations after 48 days of exposure and the correlation with whole-body hydroxyproline content was high (r = 0.824). The differences noted between 48 and 118 days were again probably due to mortality; no mortality occurred before 48 days, but by 118 days, 21% of the fish died in the 3.1 μg/l exposure and 50% died in the 6.2 μg/l exposure.

No significant effects on growth were found in fish exposed to 2,4-D DMA or DEHP (Table 3). The collagen content of bone was significantly reduced, but the hydroxyproline concentrations in collagen were either not affected or were significantly increased, whereas both collagen and hydroxyproline were decreased in fish exposed to toxaphene and Aroclor 1254. Correlation coefficients for collagen or hydroxyproline and fish weight were low (r = 0.044-0.461) in all fish exposed to 2,4-D DMA and DEHP--the exception being for brook trout exposed to DEHP (r = 0.822 and 0.786 for collagen and hydroxyproline, respectively). The reason for differing biochemical responses involving collagen and hydroxyproline with different chemicals is not clear, but this question is explored later in our discussion of vitamin C.

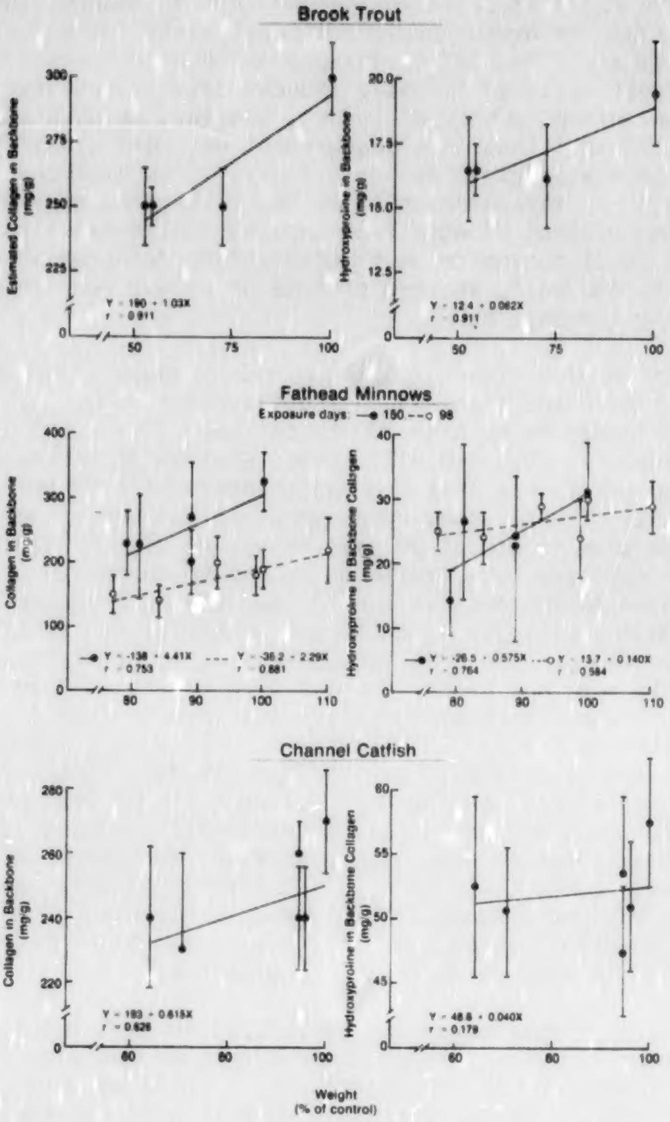


Figure 1. Relation between backbone development (vertebral collagen and hydroxyproline) and weight (expressed as % of controls) of fish continuously exposed to toxaphene. The vertical lines represent one standard deviation.

The use of collagen and hydroxyproline measurements as indicators or predictors of effects of environmental contaminants on growth of fish shows promise, but this approach has not been sufficiently studied. Measurements of hydroxyproline have an advantage over those of collagen because hydroxyproline can be directly determined in eggs and whole fry, whereas collagen is determined indirectly, except in fish that have backbones large enough for analysis. The impact of toxicants on collagen and hydroxyproline metabolism in fish appears to be greatest during early life. Young fish are growing rapidly and are generally more sensitive to toxicants that older fish. Since the present results show that the hydroxyproline and collagen contents of bone are not always directly related, both constituents should be measured when possible to facilitate toxicological interpretation. Considerable variation existed in collagen, hydroxyproline, and inorganic constituents of bone in control fish of the same species in different studies. This variation poses a serious problem in interpreting these characteristics as contaminant indicators under field conditions.

Significance of Decreased Collagen in Bone

Bone mineralization is accomplished by a complex mechanism involving the accumulation of phosphorus salts, and then calcium salts by immature bone (Nusgens et al. 1972). This mineralization process can occur independently of the development of the collagen matrix, i.e., the organic substrate of bone is not believed to be necessary for initiation of mineralization. All the organic chemicals tested apparently depressed collagen synthesis and lowered concentrations of vertebral collagen. The resulting effect of the test chemicals on bone composition was an increase in the ratio of minerals to collagen (Table 3). Mineralization of bone is a natural process, but the organic chemicals studied appear to greatly enhance this process, as seen, for example, in brook trout exposed to toxaphene (Fig. 2).

Calcium metabolism may have been affected, since its concentration in the vertebrae of all species of fish tested (and with all chemicals) increased more than could be accounted for by the concomitant decrease in collagen. Phosphorus metabolism did not appear to be affected; its concentrations in bone remained relatively constant regardless of toxicant concentrations. Exceptions to this were increases in phosphorus similar to those of calcium in brook trout exposed to toxaphene and in fathead minnows exposed to 2,4-D DMA. However, further studies on mineral metabolism are needed to determine whether calcium and phosphorus in bone are specifically affected by organic toxicants.

The reduction of vertebral collagen has a potential debilitating effect on fish; increased mineralization and brittleness of vertebrae weakens their backbones. Many fathead minnows and channel catfish x-rayed after exposure to toxaphene had broken or deformed backbones (Fig. 3,4). In catfish, portions of vertebrae were missing or compressed, especially in the anterior and posterior regions. In natural waters, the affected fish would almost certainly be less capable of competing for available food and habitat or avoiding predators. Investigations are needed to specifically determine

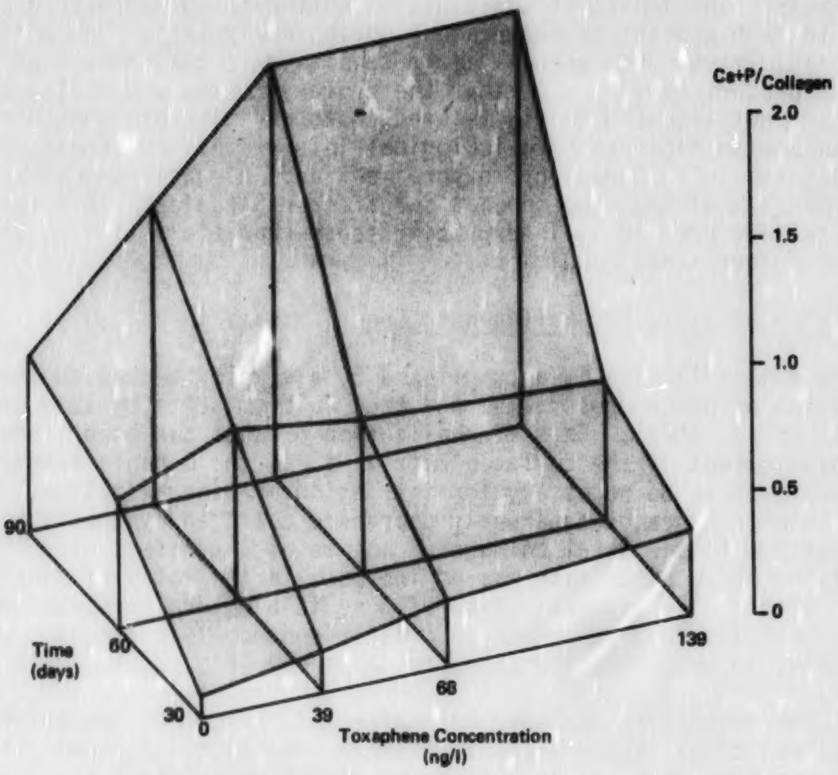


Figure 2. Effect of toxaphene on the backbone composition of brook trout fry exposed for up to 90 days after hatch. The composition is depicted as the ratio of calcium and phosphorus concentrations to collagen in dried vertebrae.

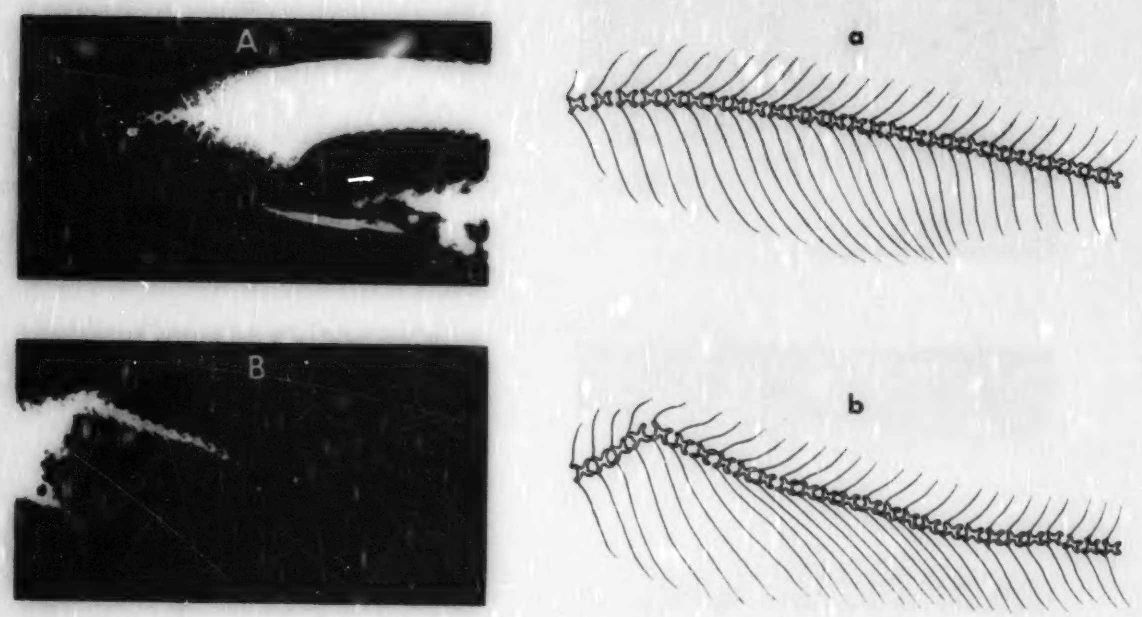


Figure 3. X-rays and schematics of backbones of 150-day-old fathead minnows: Aa, control fish; Bb, fish exposed to 94 ng/l of toxaphene.

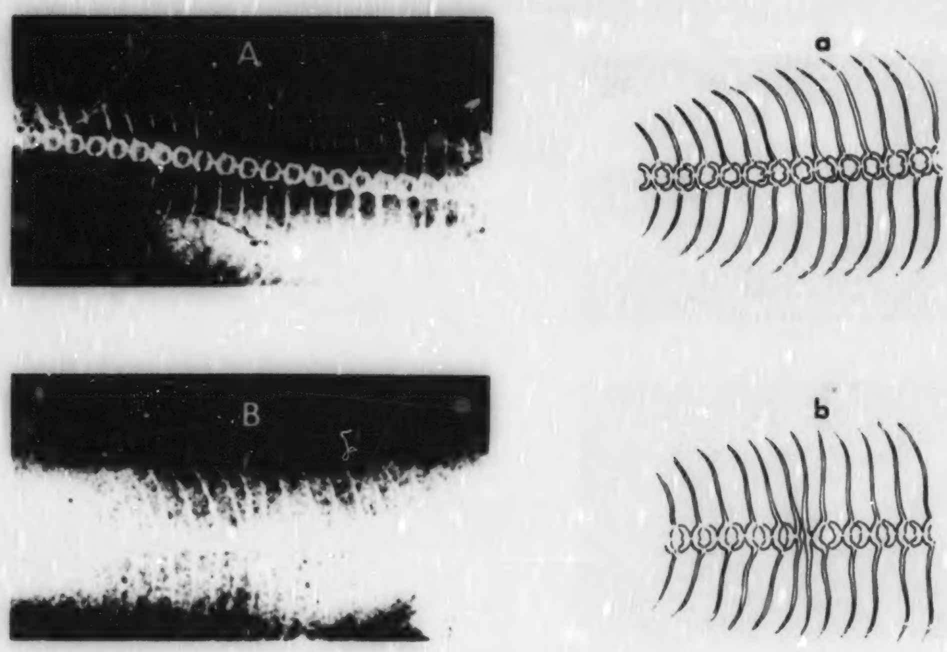


Figure 4. X-rays and schematics of backbones of 90-day-old channel catfish: Aa, control fish; Bb, fish exposed to 72 ng/l of toxaphene.

at what level alterations of organic to mineral ratios in vertebrae become a negative factor in fish health and survival.

Role of Vitamin C in Detoxication and Collagen Formation

Vitamin C is a cofactor in the hydroxylation of drugs and chemicals in the liver of mammals (Axelrod et al. 1954, Levin et al. 1960, Street et al. 1971, Wagstaff and Street 1971). It is also essential to collagen formation by way of the hydroxylation of proline and lysine into hydroxyproline and hydroxylysine (Barnes 1969, Barnes et al. 1970, Mussini et al. 1967, Peterkofsky 1972). However, vitamin C is an essential and limiting dietary nutrient in fish because fish are unable to synthesize it (Chatterjee 1973, Wilson 1973). Inasmuch as these two hydroxylation processes may compete for available vitamin C in fish, Mayer et al. (1977) investigated the effects of toxaphene on the distribution of this vitamin in liver and vertebrae of channel catfish.

Body weight, vertebral collagen, and vitamin C in liver and vertebrae were determined for fish after 90 and 150 days of exposure to 37-475 ng/l of toxaphene and fed diets containing 63, 670, or 5,000 mg/kg of vitamin C. Growth was significantly reduced in fish fed the diet containing 63 mg/kg of vitamin C and exposed to the three highest concentrations of toxaphene (Fig. 5,6). Growth was also reduced in fish fed 670 mg/kg of vitamin C and exposed to 475 ng/l of toxaphene. No change in collagen concentrations of vertebrae were observed in fish exposed for 90 days, except for those exposed to 37 ng/l of toxaphene and fed the lowest vitamin C diet. However, after 150 days of exposure, all concentrations of toxaphene significantly reduced vertebral collagen levels in fish fed the diet containing 63 mg/kg of vitamin C; the three highest toxaphene concentrations reduced collagen levels in fish fed 670 ng/kg of vitamin C; and only the 475 ng/l toxaphene concentrations decreased collagen in fish fed 5,000 mg/kg of vitamin C.

The ratio of vitamin C concentration in liver to that in vertebrae increased most in fish exposed to toxaphene for 90 days and fed the lowest vitamin C diet (Fig. 5). The slopes of the regression curves for these ratios decreased with increasing dietary vitamin C to almost no perceptible effects in the highest vitamin C diet. A similar trend was observed in ratios for fish fed the medium and high vitamin C diets after 150 days exposure to toxaphene (Fig. 6), but effects on the vitamin C content of liver and vertebrae were more pronounced than at 90 days. After 150 days, vitamin C was low in the vertebrae of all fish, including the controls, fed the diet containing 63 ng/kg of vitamin C. This response in the controls was probably due to the chronic effects of the low vitamin C diet itself.

The exposure of fish to an organochlorine contaminant, such as toxaphene, may markedly reduce the amount of collagen in the vertebrae, possibly because the use of vitamin C by the liver in hydroxylative detoxication mechanisms is increased, as indicated by induction of liver enzyme activity (Mayer et al. 1977). Vitamin C in vertebrae was reduced as much as 50%, and this reduction in bone probably inhibits the formation of hydroxyproline and hydroyxylysine from proline and lysine, which in turn reduces collagen formation.

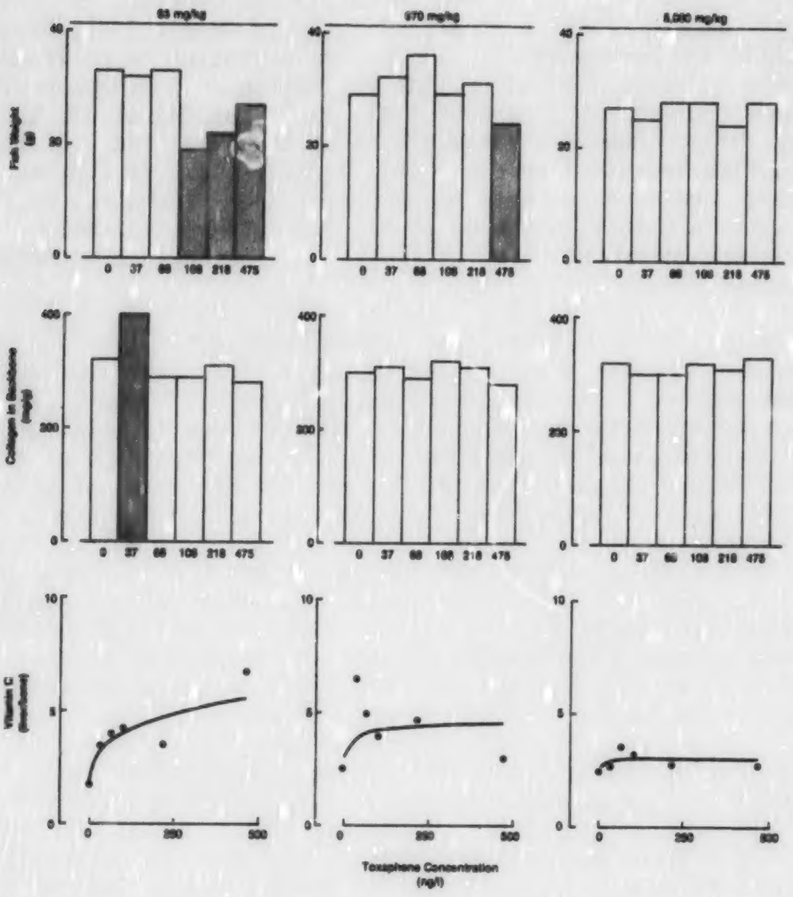


Figure 5. Toxaphene-vitamin C interaction effects on growth, vertebral collagen, and vitamin C distribution in liver and vertebrae of channel catfish fingerlings continuously exposed to toxaphene for 90 days. Shaded areas indicate values significantly different (P < 0.05) from the controls, and vitamin C concentrations in liver and vertebrae are expressed as ratios.

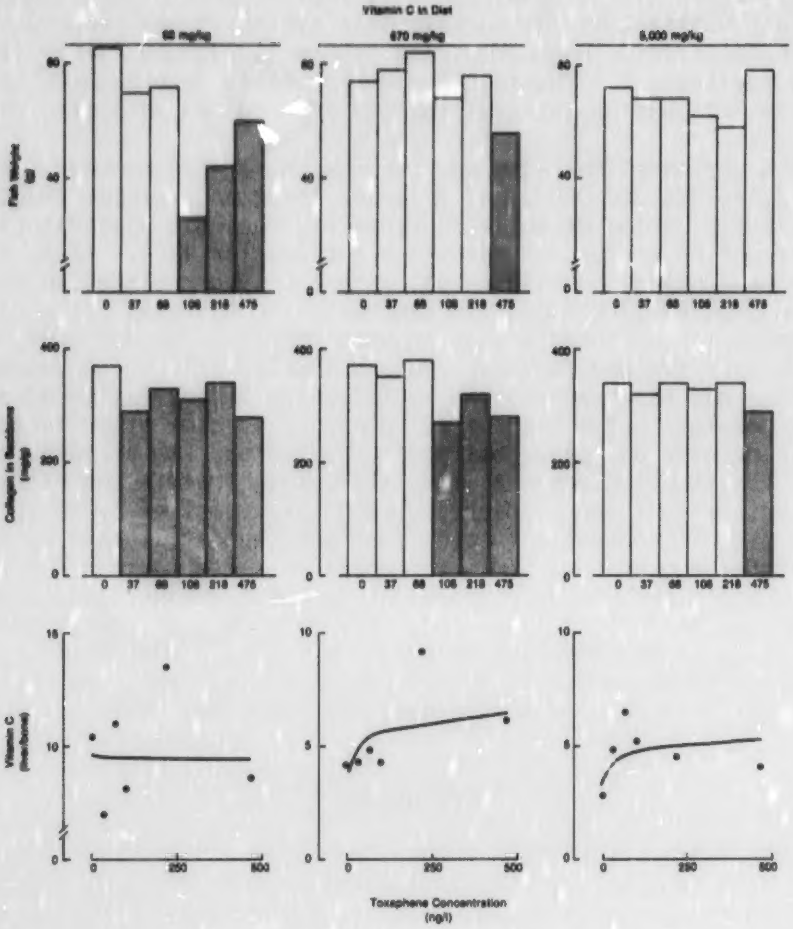


Figure 6. Toxaphene-vitamin C interaction effects on growth, vertebral collagen, and vitamin C distribution in liver and vertebrae of channel catfish fingerlings continuously exposed to toxaphene for 150 days. Shaded areas indicate values significantly different ($P < 0.05$) from the controls, and vitamin C concentrations in liver and vertebrae are expressed as ratios.

In addition, the increased use of vitamin C in liver detoxication processes may have a direct adverse effect on growth and development of fish. Dieter (1968) reported that vitamin C stimulates the conversion of folic acid to the metabolically active folinic acid, and the folic acids and vitamin B_{12} are necessary for growth of many higher animal species, especially during embryogenesis where tissue development is rapid (Cantarow and Schepartz 1962). Also, nutrient utilization for nucleic acid synthesis was reduced in animals deficient in folic acid (Huennekens and Osborn 1959), and Dieter (1968) hypothesized that vitamin C might function during early developmental processes by indirectly influencing the availability of required metabolic cofactors.

Although vertebral collagen was reduced in fathead minnows continuously exposed to 2,4-D DMA and DEHP, and in brook trout and rainbow trout exposed to DEHP, this inhibition of collagen synthesis does not appear to involve the same mode of action as that of toxaphene and Aroclor 1254. Also, changes in collagen concentration and fish weight were poorly correlated in fish exposed to 2,4-D DMA or DEHP (Table 3). In contrast, the hydroxyproline content of collagen tended to increase in fish exposed to 2,4-D DMA and DEHP, whereas it decreased in fish exposed to toxaphene and Aroclor 1254. The increase of hydroxyproline may have been caused by increased hydroxylation of proline in collagen, incomplete catabolism of collagen, or to some other factor. However, we did observe an apparent increase in catabolism of total body proteins in rainbow trout exposed to DEHP (Fig. 7). Protein concentrations in whole fish were significantly decreased and the amount of hydroxyproline in relation to protein content of whole fish increased at 24 days of exposure. After 60 days of exposure to DEHP, vertebral collagen decreased from 187 to 125 mg/g and the amoung of hydroxyproline in collagen increased from 33 to 38 mg/g as DEHP concentrations increased. The earlier differences of hydroxyproline in collagen had disappeared by 90 days (Table 3). Similar effects on protein metabolism may occur in fish exposed to 2,4-D DMA, and may in part explain the difference in responses observed among the various chemicals tested.

CONCLUSIONS

Biochemical characteristics such as collagen and hydroxyproline concentrations in bone can be used (within limits) as indicators or predictors of growth in fishes exposed to organochlorine contaminants. Measurements of those variables may shorten chronic toxicity tests. Although growth can be directly related to collagen and hydroxyproline metabolism in fishes, the mechanism by which growth is reduced is not known. Other biochemical proccesses requiring vitamin C may also be affected when large amounts of the vitamin are used by the liver in detoxification of organochlorine contaminants through microsomal hydroxylative enzymes. Chemicals such as 2,4-D DMA and DEHP can also cause a reduction in vertebral collagen without a reduction in growth, at least within the limitations of these studies. The manner in which 2,4-D DMA and DEHP is metabolized and affects fish may be an important consideration in defining the differences observed between these chemicals and organochlorine chemicals such as toxaphene and Aroclor 1254. However, the reduction of vertebral collagen can be a debilitating factor in

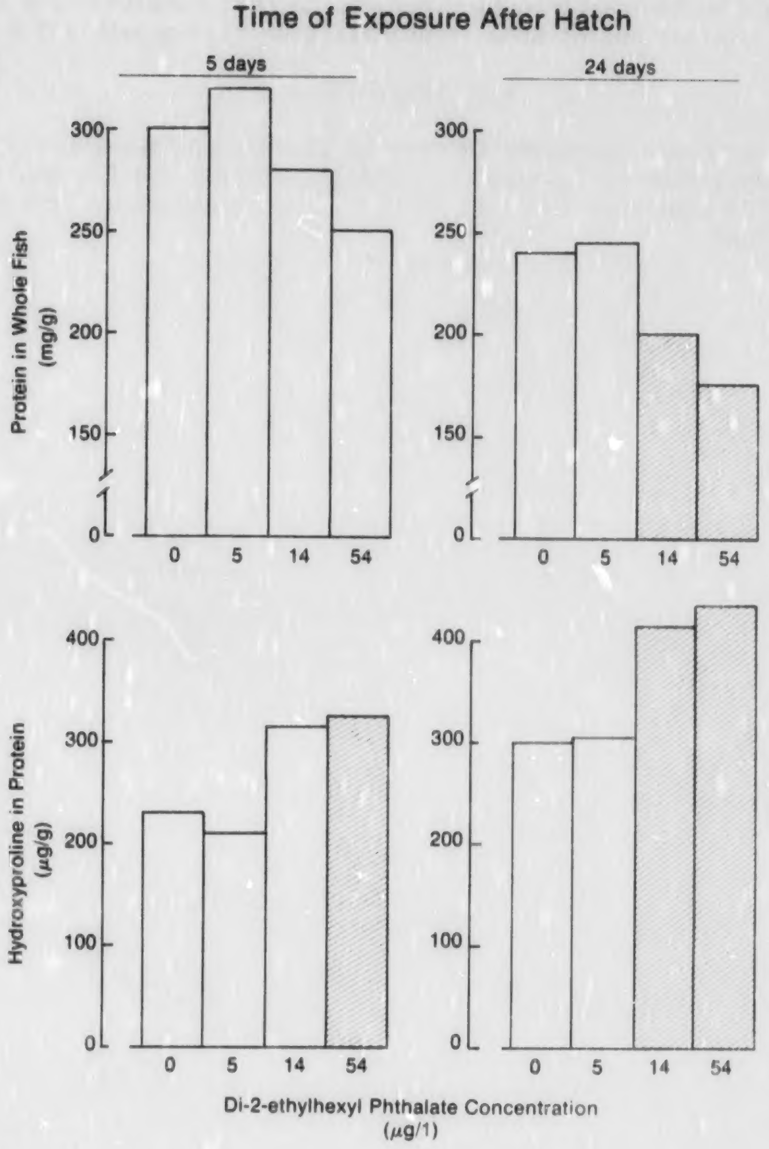


Figure 7. Di-2-ethylhexyl phthalate effects on the protein and hydroxyproline content of rainbow trout fry continuously exposed for 5 and 24 days after hatch. Shaded areas indicate values significantly different ($P < 0.05$) from the controls.

itself by increasing the probability of structurally weakened vertebral columns, and the biochemical processes related to that condition are useful in toxicological evaluations of organic chemicals on fish growth and development.

ACKNOWLEDGMENT

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EFFECTS OF SHORT-TERM EXPOSURES TO TOTAL RESIDUAL CHLORINE ON THE SURVIVAL AND BEHAVIOR OF LARGEMOUTH BASS (*Micropterus salmoides*)

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ABSTRACT

Largemouth bass were subjected to short-term exposures of total residual chlorine. Two different time-toxicant concentration curves similar to those of chlorinated discharges from power generation plants were used as models for the tests. One discharge curve (referred to as the square exposure) was characterized by a rapid rise in toxicant concentration to a plateau level, followed by a rapid decline in concentration after toxicant introduction was terminated. The second discharge curve (referred to as the spike exposure) was characterized by a rapid rise to a peak toxicant concentration, immediately followed by a rapid decline in toxicant concentration. Acute toxicity tests included a comparison of the effects of square and spike exposures, and comparative tests of the effects of square exposures of varying frequency and duration. Fish behavior was observed during acute and sublethal square and spike exposures.

There were no obvious differences in acute toxicity between the two types of exposures when mortality (in probits) was plotted against the areas under the time-concentration curves. The same results were obtained in tests of one and two 90-min. exposures, and for one 90-min. exposure and one 150-min. exposure. Thus, measurement of the areas under the time-concentration curves are a useful

means of studying effects of different kinds and durations of exposures and different exposure frequencies.

Bass exhibited several behavioral changes during the acute toxicity tests. Many behavioral responses occurred in sublethal tests of square and spike exposures. The behavioral changes caused by acute and sublethal exposures probably are detrimental to the well-being and survival of the fish in the field.

INTRODUCTION

Intermittent (recurrent) chlorination of cooling waters is a common method employed to remove organisms from heat exchangers in power generation plants. Only 10 percent of the power plants in the U.S. chlorinate on a regularly programmed basis (Brungs 1976). Considerable variation exists between power plants with regard to the duration, frequency, and amounts of chlorine introductions. Additional differences between the discharges from power plants include temperature, water quality (e.g. heavy metal contamination), toxicant concentrations at the points of discharge into receiving waters, and the forms of the residual chlorine. The heated effluents from power plants are discharged into rivers, lakes, or estuaries, but the effluents may pass through channels or ponds before discharge into receiving waters.

The toxicity of residual chlorine to aquatic organisms under conditions of continuous exposure does not provide reliable information on the potential toxicity of intermittent exposures to residual chlorine. Extrapolation of laboratory results to the field situation is most appropriate when aquatic animals are exposed to short-term introductions of residual chlorine over an adequate time.

Based upon literature on the toxicity of residual chlorine to aquatic organisms kept under continuous exposure to constant toxicant concentrations in laboratory experiments, considerable concern has developed regarding the potential toxicity of short-term introductions of residual chlorine to the organisms. The short-term exposures present a number of special problems of analysis and comparison of laboratory and field data. Major problems facing investigators include the effects of the fluctuating toxicant concentrations discharged into receiving waters, and effects of the variations in duration and frequency of the introductions. As a means of dealing with some of these problems, investigators have often exposed fish to constant concentrations of residual chlorine in laboratory aquaria for short periods of time (McLean 1973; Stober and Hanson 1974). Such tests do not mimic the fluctuating toxicant concentrations to which fish are exposed in the field. Other investigators have exposed fish to fluctuating toxicant concentrations and have calculated LC50's on the bases of the mean or peak concentrations of the exposures (Brooks and Seegert 1977; Heath 1977). Basing LC50's on mean concentrations probably is a useful method for comparing different experiments when the durations and frequencies of the exposures are known. Acute toxicity data based on peak concentrations, i.e. when the toxicant attains a peak concentration and then declines in concentration rapidly, are not comparable, however, unless the time-toxicant relationships are identical.

It appears that comparisons made on the basis of mean exposure concentrations to fish for varying periods would be useful in developing an understanding of the toxicity to aquatic organisms in intermittent discharges of chlorine. However, the variable characteristics of intermittent chlorinated discharges and the array of possible field conditions to which aquatic organisms could be exposed would undoubtedly lead to nearly endless experimentation.

One way to deal with the complexity of the field conditions is to compare the exposures in acute toxicity tests on the basis of the areas under the time-concentration curves. We assumed toxicity was constant for a given area under the time-concentration curves without regard for the shape of the curve, the exposure frequency (assuming no recovery between exposures), or the duration of the exposures. The first objective of our work was to explore the utility of the area concept as it applied to comparing or predicting the toxicities of intermittently chlorinated power generation plant discharges.

Secondarily, we observed the behavioral changes of fish during exposures to short-term introductions of residual chlorine in laboratory aquaria. This work was initiated because several investigators during fieldstudies noticed major behavioral changes in fish subjected to short-term exposures to chlorine (e.g. Basch and Truchan 1976). Erratic behavior might result in physical damage to fish by reducing the ability to avoid obstacles or increasing the susceptibility to predation.

METHODS

Largemouth bass were collected from a farm pond near Corvallis, Oregon, in early June, 1975. Fish were acclimated to the laboratory conditions for at least one month prior to testing and were fed Oregon Moist Pellets daily. Feeding was discontinued one day before the tests. The fish were maintained under the photoperiod regime for this region.

Acute toxicity bioassays were carried out with standard 45-l. glass aquaria, initially containing 40 l. of water. Dilution water and chlorine solutions were introduced to the aquaria through two PVC manifolds, each 3.81 cm in diameter. Rapid changes of the chlorine concentrations in the aquaria were achieved by reducing the water volume to 30 l. (20 cm maximum depth) and by appropriately manipulating dilution water and toxicant flows.

Chlorine stock solutions were made in a Mariotte bottle by mixing sodium hypochlorite and well water. The well-water supply was located at the laboratory. Average water quality characteristics of the well water were: dissolved oxygen 7.4 mg/l, hardness 128 mg/l, total alkalinity 148 mg/l, pH 7.94, and temperature 24.3 C. Grab samples of chlorine test solutions were analyzed using a Wallace and Tiernan amperometric titrator. Free residual chlorine averaged 97.04± 1.23 percent* of the measurable total residual chlorine (TRC) in the test solutions.

Each experiment was completed within one week. In each acute toxicity test, six bass of nearly equal size were acclimated for 30 min. to the test conditions before the toxicant was introduced. Concentrations of chlorine were measured at 2-10 min. intervals for the duration of each exposure, when the toxicant was present in the aquarium. In 96-hr. acute toxicity tests fish were exposed to chlorine and then maintained in fresh water for the duration of the test. Mortalities were recorded daily; dead fish were removed. At the end of each test, fish were dried at 70 C. for 7 days and weighed.

In these studies two types of time-toxicant concentration curves (referred to as square and spike exposures) were used as models (Figure 1). These curves represented the extremes of those found in the power plant effluents at the points of discharge into receiving waters (G. Nelson, EPA, personal communication). The square exposures were produced by adding the toxicant to the aquaria at constant rates for predetermined periods. Chlorine concentrations reached a plateau level 20 min. after initiating the toxicant flow. With one exception the toxicant flow was terminated at 60 min. and the toxicant was completely flushed from the aquaria after an additional 30 min. (90 min. total exposure time). Spike exposures were characterized by a rapid rise to a peak toxicant concentration, followed immediately by a rapid decline. In bioassays using the spike exposures both toxicant and dilution water flows were manipulated to achieve the desired curves. High and low spikes (relative to each other) were used in some tests, only the low spikes in other tests. The high spike exposure peaked in concentration 5 min. after initiation of toxicant flow, the low spike exposure peaked at 22 min. Total exposure times were 51 min. for the high spikes and 63 min. for the low spike exposures.

Acute toxicity tests at our laboratory have shown that fish weight can affect the tolerance of coho salmon to residual chlorine (Larson et al. 1977). The bass used in the present work were not of uniform weight. Preliminary tests were conducted in mid-July to determine if body weight affected the tolerance of the bass to short-term exposures to chlorine. Two weight groups were tested, one being 3.87± .15, the other 5.93± .28 g/fish, dry weight. Groups of each weight class were exposed to one 90-min. square exposure. The 96-hr. LC50 for the two classes differed by approximately 1.2 mg/l TRC (mean plateau concentration). Smaller fish were more sensitive. On the basis of these preliminary results, fish weight was standardized in each experiment, although the weights varied from test to test (Table 1).

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* ± 1 standard deviation

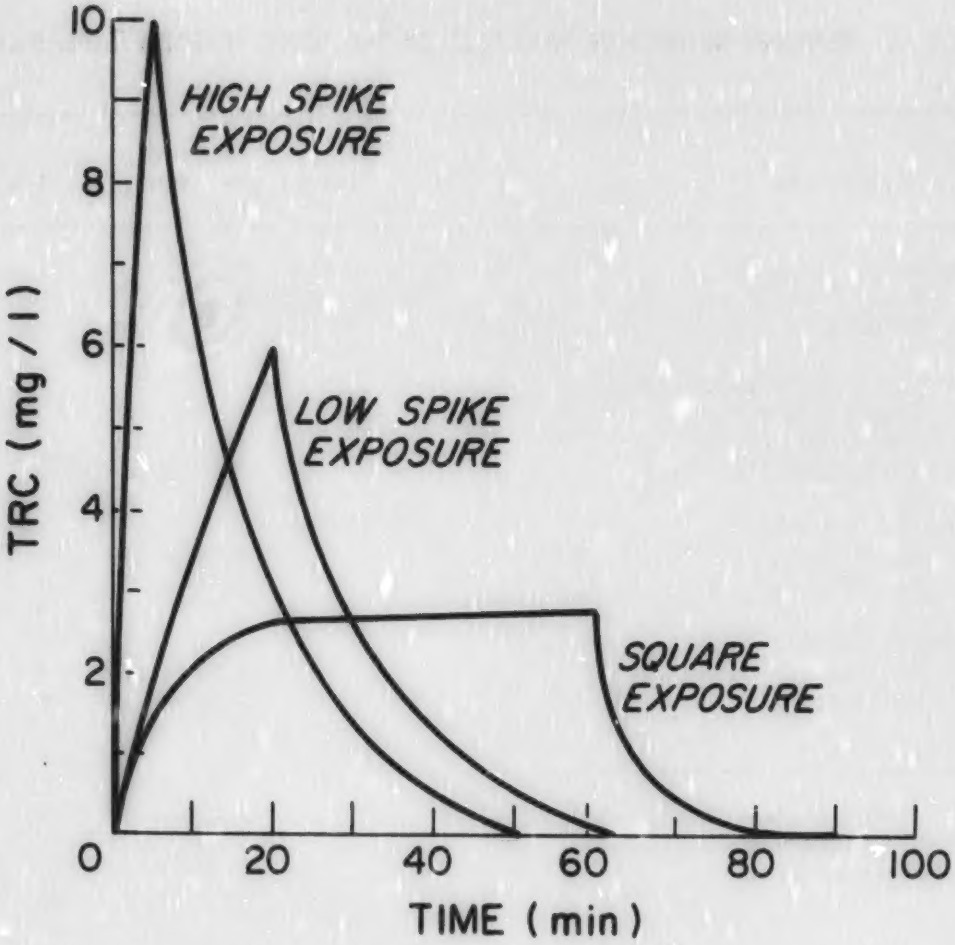

Figure 1. Examples of the time-concentration relationships of the square exposure and the high and low spike exposures with total residual chlorine (TRC).

TABLE 1. MEAN DRY WEIGHTS OF BASS USED IN THE ACUTE TOXICITY EXPERIMENTS

Experiment	Mean dry weight per fish (g) ± 1 S.D. [a]
Body Weight[b]	
Group 1	3.87 ± .15
Group 2	5.93 ± .28
Square vs. Spike Exposures	
Square (90 min.)	8.60 ± .20
Low spike (63 min.)	8.50 ± .20
High spike (51 min.)	8.51 ± .24
Exposure Frequency[b]	
one 90 min.	6.18 ± .19
two 90 min.	6.15 ± .32
Exposure Duration[c]	
90 min.	8.92 ± .36
150 min.	8.92 ± .53

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[a] ± 1 Standard deviation
[b] 90-min. square exposures
[c] Square exposures

Groups of bass were exposed to a high or low spike exposure, or to the square exposure. The mean plateau concentrations of TRC in square exposures ranged from 2.35 to 3.32 mg/l. The peak TRC concentrations in spike exposures ranged from 8.21 to 11.93 (high spike exposures) and 5.73 to 9.06 mg/l (low spike exposures).

Effects of the three types of exposures were compared on the basis of areas under the time-concentration curves. Areas under the time-concentration curves were measured using a compensating planimeter. The curves for all tests were graphed using the following scale: a 10-min. exposure to 1 mg/l TRC equalled 5.9 cm^2.

Exposure-frequency effects were examined by subjecting some groups of bass to single 90-min. square exposures, while other groups were subjected to two such exposures. In the two-exposure groups, a 2-hr. recovery period separated the exposures.

The effect of exposure duration on survival was investigated by subjecting groups of bass to either a 90-min. or a 150-min. square exposure. For the 150-min. exposures, toxicant flow into the aquaria was terminated at 120 min.

Observations of the changes in behavior of the bass were made throughout each acute toxicity test; the majority were made during the exposure period and at 24-hr. intervals for the duration of the 96-hr. tests. Times for the first occurrence of particular behavioral responses were recorded during most exposures. After the acute toxicity studies, an experiment was conducted to determine the behavior of individual bass exposed to sublethal concentrations of chlorine. The experimental equipment and most of the procedures were identical to those described above. Two groups of bass were used, one averaging 14.63 ± .39 g, and the other 7.37 ± .62 g dry weight per fish. In most cases two fish, one from each group, were kept together in separate aquaria for at least 5 days prior to testing. The paired fish were then subjected once to either a 90-min. square exposure or a 63-min. low spike exposure, and then maintained in fresh water for the duration of the 96-hr. test. Water samples for determining TRC concentrations were obtained by siphoning test solutions from the aquaria. The range of concentrations in square exposure tests was 140 to 2422 μg/l (averages for the 20-min. to 60-min. plateau period), and that in spike exposure tests was 400 to 6980 μg/l peaks. Behavioral observations were made by an observer sitting 2 m from the test aquaria during the exposures and for 1 to 2 minutes at 24-hr. intervals thereafter.

Standard statistical methods (Sokal and Rohlf 1969) were used to perform regression analyses.

RESULTS AND INTERPRETATIONS

Mortalities usually occurred within 24-48 hours after the exposures. Dying fish turned over (belly up) or rested upright on the aquaria bottoms, and had much coagulated mucus adhering to the gills at the ends of the exposures.

The relationships between mortalities (in probits) and the areas under the square, low spike, and high spike exposure curves was examined (Figure 2). There were no obvious differences in toxicity between the three types of exposures when the areas under the curves were equal. These results suggest that within the range of experimental toxicant concentrations the shapes of the time-concentration curves were not as important as the total exposure areas under the curves.

The LC50 for bass subjected to one 90-min. exposure of chlorine was substantially greater than that for bass subjected to two 90-min. exposures when each was expressed as mean plateau concentrations (Figure 3A) or as mean concentrations for the duration of the exposures (Figure 3B). However, there were no differences in mortalities between the two types of exposures for a given exposure area (Figure 3C). Similarly, the LC50 for bass subjected to one 90-min. exposure was greater than that for one 150-min. exposure based upon toxicant concentrations (Figures 3D and E), but there was little, if any, difference between the effects of exposures that were alike on an areal basis (Figure 3F).

These results suggest that measurement of the areas under the curves is a useful approach when comparing the toxicity to largemouth bass of different types of short-term exposures of residual chlorine (mostly free residual). Furthermore, when expressed on an areal basis, the results of the experiment with one and two 90-min. exposures indicated that there was insufficient recovery of the bass during the 2-hr. rest period between the exposures to reduce the mortality associated with a given area under the time-concentration curve (sum of 2 exposures). With sufficient recovery time fewer deaths would have occurred at a given exposure area, and the response points would shift downward (Figure 3C) and to the right. If the fish had attained complete recovery between the exposures, no deaths would have resulted from the second exposure.

During the 30-min. acclimation period before each acute toxicity test, the bass swam slowly and deliberately and seldom coughed. A number of changes of behavior were observed during the tests, however. The changes usually occurred in the following sequence: (a) increases of the rates of swimming, opercular activity, and coughing; (b) reduced swimming activity near the surface of the water, i.e., positioning just under the water surface; (c) rapid swimming with thrashing at the water surface, some jumping; (d) lethargic swimming, frequent collisions with aquarium walls and other fish; (e) "bobbing," i.e., with dorsal portion of head exposed at the water surface; (f) resting on tank bottom with heavy, pulsating opercular activity, and some spurts of irregular swimming; and (g) turning over (belly up).

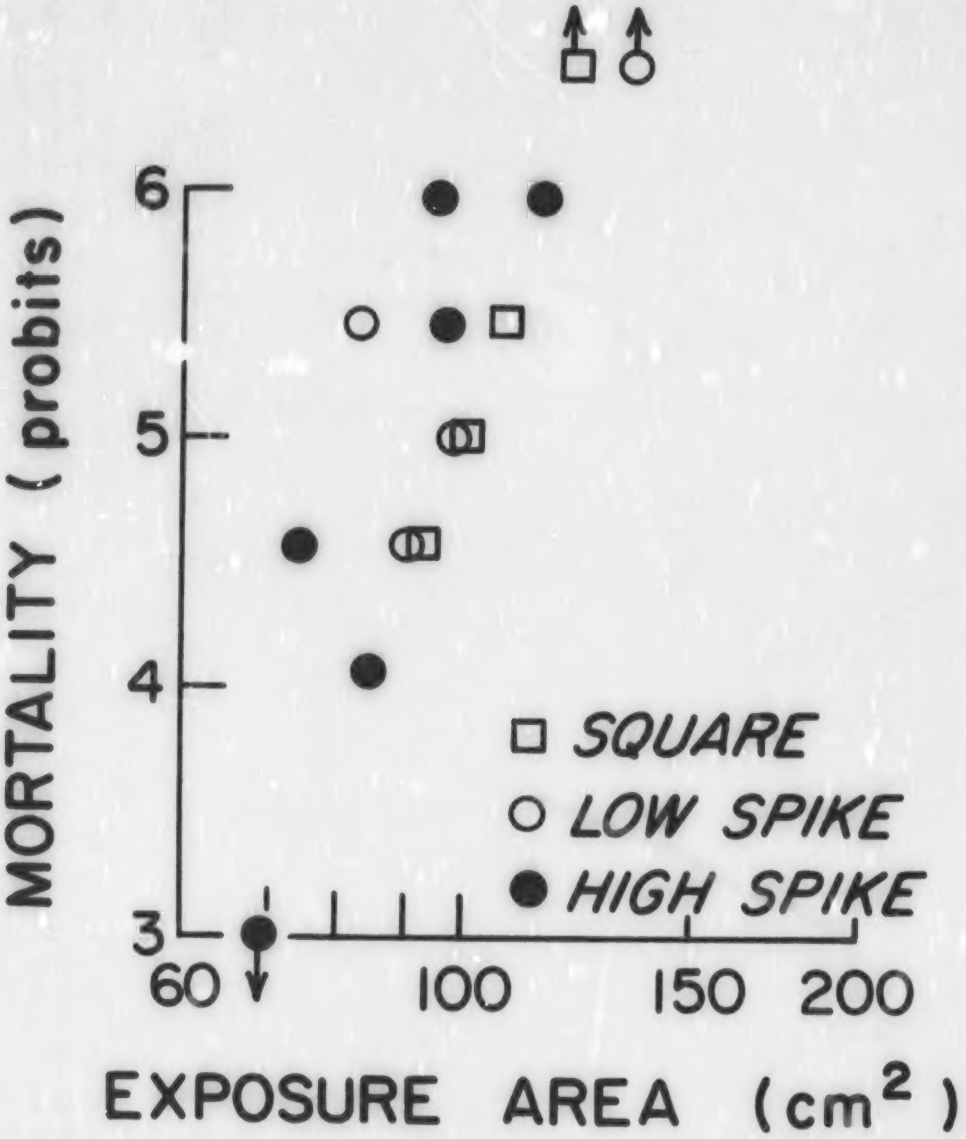

Figure 2. Relationships between mortality (in probits) and exposure area (area under the time-concentration curve) for bass subjected to square exposures and high and low spike exposures.

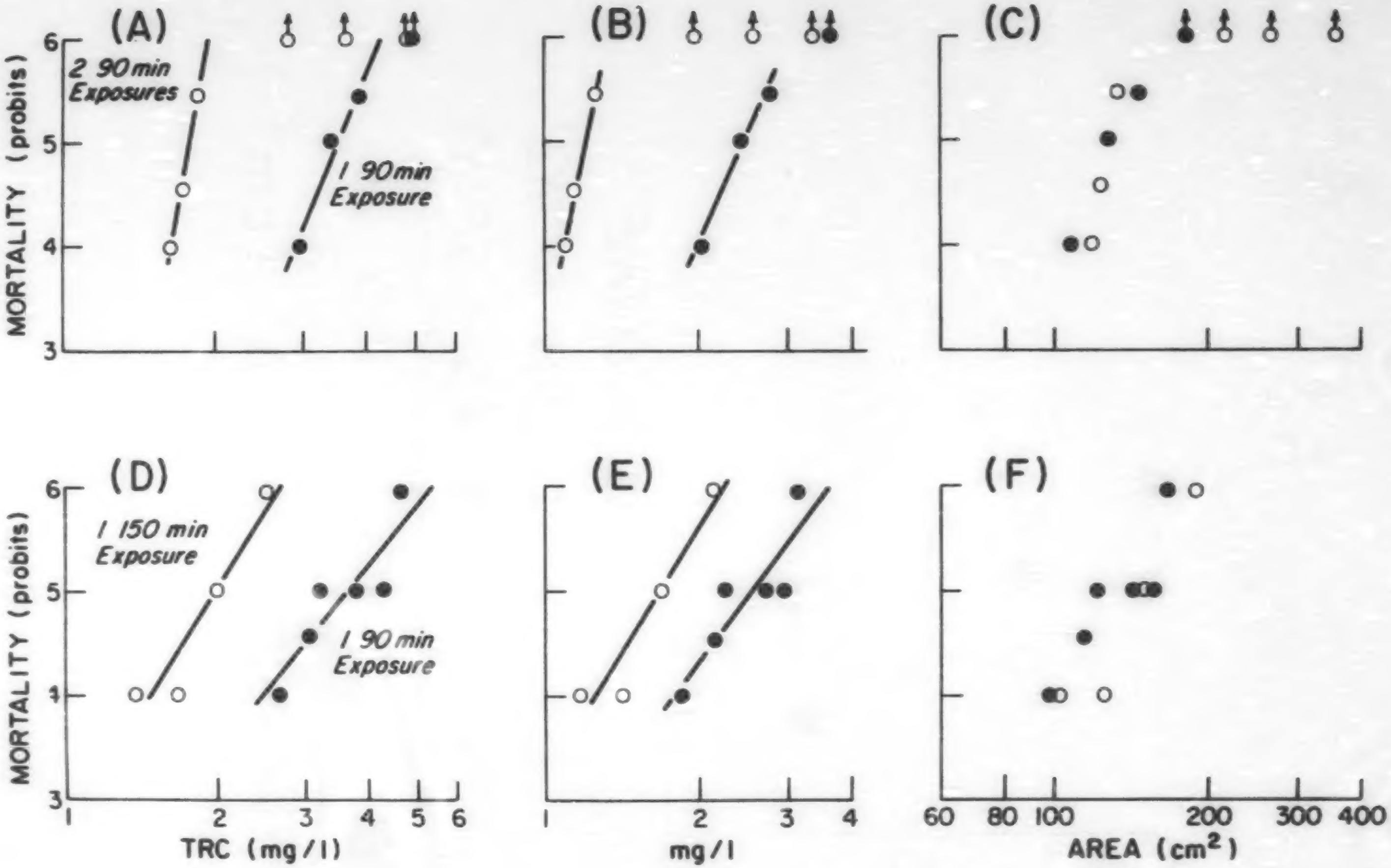

Figure 3. Relationships between mortality (in probits) and the average plateau concentration of total residual chlorine (A and D), mean concentration for the entire exposure (B and E), and area under the time-concentration curve (C and F) for bass subjected to one or two 90-min. square exposures, or to one 90-min. or one 150-min. square exposure.

Despite the consistency of the behavioral sequence, individual kinds of behavior often failed to occur. This absence was particularly evident in tests of low or high TRC concentrations relative to the 96-hr. LC50. Fish that did not die usually exhibited normal behavior within 24 hours after the exposures.

Judging the time to first occurrence of each kind of behavior was subjective but the time appeared to decrease as toxicant concentrations increased. Only the first occurrence in each group of fish was recorded, and it was not possible to relate the times to areas under the time-concentration curves, because the bass exhibited a particular response at different times when the curves were of different height but had the same area to the time of the response. Thus, our preliminary results were expressed necessarily as mean concentrations for the exposures to the times of first occurrence. An example of the relationship for bobbing behavior is taken from the experiment in which the 90-min. square exposure was repeated (Figure 4). The time to bobbing decreased as the toxicant concentration increased in the first exposure. Bobbing occurred earlier in the second exposure than in the first at nearly equal toxicant concentrations. However, two groups did not exhibit bobbing in the second exposure. The two groups were exposed to acutely toxic concentrations in the first exposure, and some of the fish were on the bottoms of the aquaria at the start of the second exposure. The other fish in the aquaria were active, but their behavioral changes progressed quickly through the above sequence during the second exposure and the bobbing behavior was skipped completely (i.e., was never observed).

The behavioral tests of sublethal concentrations of TRC in square and spike exposures were conducted to estimate the range of concentrations at which several of the kinds of behavior mentioned above first occurred. No deaths occurred in these tests. At the highest sublethal concentrations the sequence of changes of behavior was consistent with the results of acute toxicity tests, except that turning over did not occur. The thresholds of occurrence of the behavioral changes were difficult to determine, but nearing the surface occurred at a smaller exposure area than did thrashing, lethargic swimming, on bottom, and bobbing (Figure 5). The results of this test are important because the five kinds of behavior occurred at sublethal toxicant concentrations.

DISCUSSION

The results of this study have shown the 96-hr. LC50 for largemouth bass subjected to short-term exposures to TRC was influenced by fish weight and by exposure duration and frequency. No obvious differences in mortality were found between groups of bass subjected to square or spike exposures to free residual chlorine when the areas under the time-concentration curves were equal. This aspect needs further investigation, however, since the TRC discharged from power plants may range from mostly combined residual chlorine to mostly free residual. Furthermore, the species composition of TRC may change during passage downstream in rivers from the points of effluent discharge (G. Nelson, EPA, personal communication).

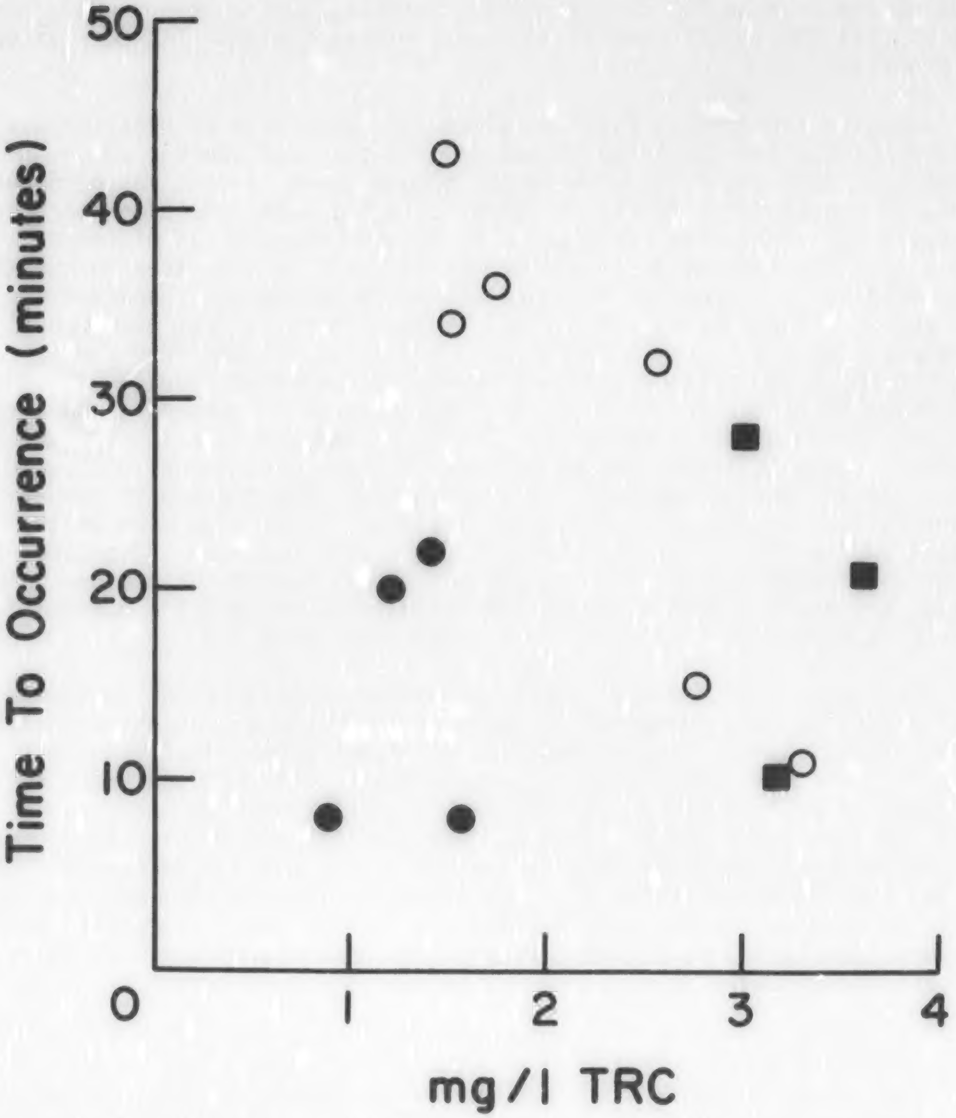

Figure 4. Relationships between the time to first occurrence of bobbing and the mean concentration of total resiudal chlorine in the aquaria until the time of first occurrence during the experiment with one and two 90-min. square exposures. Symbols: ■ - one exposure only; ○ - first of two successive exposures; and ● - the second exposure.

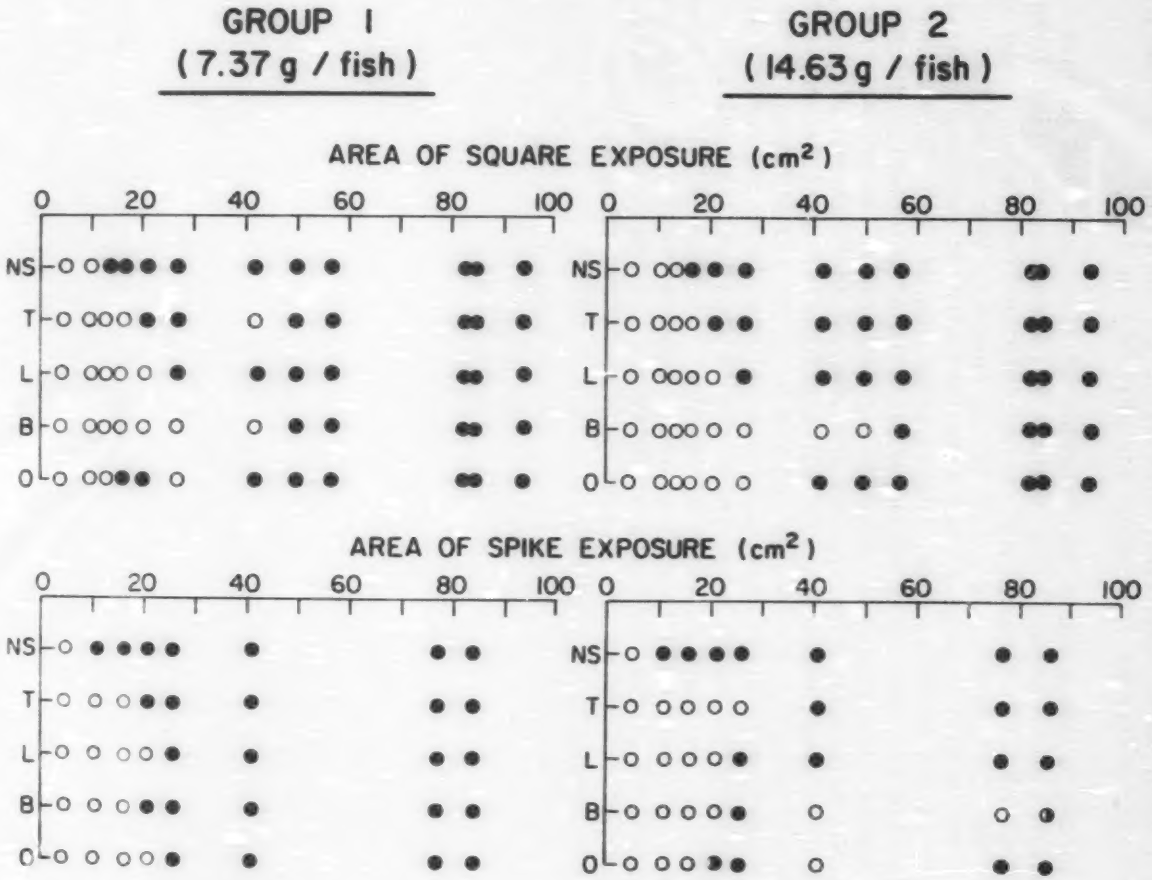

Figure 5. Relationships between exposure area and occurrence of five behavioral changes in two weight groups of bass subjected to square and spike exposures. Symbols: NS - near the surface; T - thrashing; L - lethargic swimming; B - bobbing; and O - on bottom.

The areal approach appears to be a valuable method for evaluation of the toxicity of chlorine over a wide range of laboratory test conditions, with varying exposure frequencies and durations. Using this approach, the recovery of fish between exposures can be compared directly with situations without recovery in a quantitative manner. This approach may not be valid, however, when chlorinated power plant discharges are contaminated with compounds (e.g., heavy metal complexes, Dickson et al. 1974) that have metabolic sites of activity different from those for chlorine in fish.

Very little is known about the behavior of fish subjected to intermittent exposures to chlorine in the field. Basch and Truchan (1976) showed that alewife avoided discharge plumes during chlorination, but returned when chlorination was terminated. In other tests, however, they noted that salmonids in chlorinated discharge plumes sometimes died or exhibited considerable stress at the water surface. Avian predation of fish floundering at the water surface has been observed below outfalls of chlorinated discharges from power plants (Brungs 1976). Studies are required to determine under what conditions particular fish species are trapped in discharge plumes, and more information is needed on the cumulative effects of short exposures to TRC of different species compositions on the behavior and survival of fish, even those fish able to avoid the chlorinated plumes as in the above example. Considerable attention should be given to the influence of thermal acclimation on fish on the acute toxicity of residual chlorine and on the behavior of the fish in chlorine solutions at elevated temperatures. The development of an understanding of the influence of behavioral changes on the survival and well-being of fish in waters receiving intermittent discharges of chlorine seems particularly appropriate.

ACKNOWLEDGMENTS

We wish to thank Dr. Charles E. Warren for his encouragement and guidance throughout these studies. Dr. P. Doudoroff reviewed the manuscript and offered many valuable comments and suggestions. His interest in our work was sincerely appreciated. Staff members and graduate students at the Oak Creek Laboratory of Biology kindly gave their time to discuss certain aspects of the results and helped with laboratory experiments. Their efforts were appreciated.

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AN APPROACH FOR STUDYING THE EFFECTS OF MIXTURES OF ENVIRONMENTAL TOXICANTS ON WHOLE ORGANISM PERFORMANCES

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ABSTRACT

An extensive methodology has been developed to evaluate the toxicity of individual environmental pollutants for a variety of test animals; however, an approach is needed to study the possible interactions of toxicants found together in the environment. A promising model has previously been proposed for predicting quantal (all or none) responses of organisms to mixtures of two or more toxicants. In our laboratory, toxicity studies using the common guppy, *Poecilia reticulata*, as a test organism have demonstrated the utility of this model for predicting their lethal response to a variety of toxicant mixtures. The usefulness of this approach to environmental toxicity problems is evaluated in terms of its applicability to sublethal studies. The model under investigation and results from experiments studying the effects of copper, nickel and their mixture on the gross growth efficiency, relative growth rate, and food consumption of guppies are discussed.

INTRODUCTION

An extensive methodology has been developed for evaluating the effects of discrete environmental toxicants on a variety of test organisms; however, when environmental pollution does occur several toxicants are usually present simultaneously. The recognition of this situation by environmental toxicologists and those responsible for assessing the potential hazards of man-made pollutants has generated considerable interest in developing approaches for evaluating the effects of mixtures of environmental toxicants. Sprague (1970) in his series of papers on the measurement of pollutant toxicity to fish reviewed some of the approaches and the results of previous studies assessing the joint toxicity of aquatic pollutants.

Several years ago, primarily as a result of conversations with Pete Doudoroff, Charles Warren, and others at our laboratory, we became interested in this problem and initiated a program to develop and empirically evaluate an approach for studying the effects of multiple toxicants on the whole organism performances of fish.

We recognized as others have (Plackett and Hewlett 1948) that only pharmocological studies on the modes of action of toxicants applied separately and jointly can definitively determine the type of interaction between them. However, the primary actions (the underlying processes by which toxicants initiate alterations in some pre-existing physiological or biochemical process) of toxicants has been elucidated in only a few cases. Even in these cases it can probably be expected that the more a presumed action is studied the more likely it will be found to be an effect, the sequence of biochemical and physiological events that are initiated by the action of a compound (Fingl and Woodbury 1965).

Given the difficulty and uncertainty in determining the primary mechanisms of action of toxicants, the classical pharmacological approach for evaluating the toxicity of compounds involves studying the relationship between the concentration of a toxicant and the effects it produces. The selection of an appropriate effect for evaluating the toxicity of a compound depends on the objectives of the toxicologist. Lethality is often used as a starting point for studying the toxic properties of a pollutant. Therefore, it is not surprising that most studies on the joint toxicity of environmental toxicants have been on quantal responses (all or none) - primarily death. However, to insure the success of organisms in nature, it is also necessary to study the effects of toxic substances on such whole organism performances as growth, reproduction and behavioral responses.

Plackett and Hewlett (1948) suggested that the mathematical examination of the concentration mortality curves for individual toxicants may indicate the types of combined effects that occur when the toxicants are present simultaneously. As a first step for evaluating the effects of multiple toxicants on whole organism performances, we based our approach on aspects of various models originally presented by Bliss (1939) and Plackett and Hewlett(1948) for quantal response data. Using their approach, Anderson and Weber (1977) were able in most cases to predict the effects of mixtures of selected environmental toxicants on the survival of guppies (*Poecilia reticulata*). Based on these results we designed a series of experiments to evaluate the applicability of the approach to graded (sublethal) responses.

The primary objective of this paper is to discuss the rationale of the proposed approach for studying both the quantal and graded responses of whole organisms to mixtures of environmental toxicants. Hypothetical dose response curves with their associated isobole diagrams are presented to illustrate the different types of toxicant interaction discussed. The results of preliminary experiments evaluating the effects of the chlorides of copper, nickel and their mixture on the growth rate, food consumption, and gross growth efficiency of juvenile guppies are presented (unpublished data).

RATIONALE

Using Bliss's paper (1939) as their point of departure, Plackett and Hewlett (1952) described rather general biological models for toxicant interactions and deduced mathematical models for each based largely upon statistical considerations. They proposed general types of toxicant interaction based on the following two-way classification scheme:

	Similar	Dissimilar
Non-interactive	Simple similar (concentration addition)	Independent (response addition)
Interactive	Complex similar	Dependent

They defined toxicant mixtures as "similar" or "dissimilar" according to whether the toxicants acted upon the same or different biological systems and as "interactive" or "non-interactive" according to whether one toxicant influenced the "biological action" of the other toxicants. "Simple similar" and "independent action" were regarded as special cases in a continuum of biological possibilities and the mathematical models proposed for complex similar and dependent were generalizations of the models proposed for "simple similar and independent action" respectively.

Their mathematical models particularly for the quantal responses to mixtures of "interactive" toxicants are very complex and require the knowledge of certain parameters which are normally unattainable when evaluating the effects of toxicant mixtures on whole organism performances. However, Hewlett and Plackett's models for "joint action" are useful for elucidating the limitations of and the assumptions required for the special cases of "simple similar and independent joint action". As a first approach to evaluating the effects of toxicant mixtures on the whole organism performances such as survival and growth, the present discussion only considers the special cases of "non-interactive" toxicant mixtures.

A multitude of terms have been suggested to describe the various types of combined toxicant effects. Ariens (1972) and Fedeli et al. (1972) reviewed the various terminologies that have been used. As Sprague (1970) and Warren (1971) point out, the nomenclature is confusing particularly since certain terms have been defined in more than one way by different authors. Furthermore, terminology describing mechanisms of toxicant action is not appropriate for studies evaluating the effects of toxicant mixtures on whole organism performances without knowledge of the action of the individual toxicants. To avoid both ambiguities in terminology and assumptions implying knowledge of sites and mechanisms of toxicant action, Anderson

(1977) introduced the terms *concentration* and *response addition* which are mathematically analogous to the "simple similar" and "independent action" defined by Plackett and Hewlett (1952).

Concentration addition is mathematically defined as the additive effect determined by the summation of the *concentrations* of the individual constituents in a mixture after adjusting for differences in their respective potencies. The primary assumption governing this type of addition is that the toxicants in a mixture act upon similar biological systems and contribute to a common response in proportion to their respective potencies. Bliss (1939) and others have assumed that if two toxicants act similarly the variations in susceptibility of individual organisms to the toxicants are completely correlated. As a consequence the dose response curves for the components and the mixture are parallel. This has been observed for some toxicant mixtures; however, Plackett and Hewlett (1952) presented examples of chemically related insecticides which gave non-parallel lines. They and other toxicologists (Ariens and Simonis 1961; Casarett 1975) have stated, and we believe rightfully so, that parallelism and hence complete correlation of individual susceptibilities is not a necessary prerequisite for this type of addition.

In cases where the dose response curves for the individual toxicants in a mixture are parallel, a dose response curve for the mixture can be calculated based upon the assumption of concentration addition. With the regression equations for the individual toxicants in the form of $y = a + b \log x$ (where y is the % response to each toxicant and x is its concentration), the regression equation for a binary mixture can be represented by (Finney 1971):

$$y_m = a_1 + b \log (\pi_1 + p\pi_2) + b \log Z \qquad (1)$$

where,

y_m = % response to the mixture
a_1 = y intercept of the first toxicant
b = common slope
π_1 = proportion of the first toxicant in the mixture
π_2 = proportion of the second toxicant in the mixture
p = potency of the second toxicant relative to the first
Z = concentration of the mixture

This equation can be readily adapted to represent mixtures containing more than two toxicants. It should be noted that equation (1) for concentration addition is similar in principle to the toxic unit method used by Lloyd (1961), Brown (1968) and others. Whereas the toxic unit method measures the toxicity of mixtures only at particular levels of response (LC10, LC50, etc.), equation (1) incorporates the entire dose response curve.

Response addition is the additive effect determined by the summation of the *responses* of the organism to each toxicant in a mixture. This form of addition is based on the assumption that the toxic constituents of a mixture act upon different biological systems within the organism. Each organism in a population is assumed to have a tolerance for each of the toxicants in a mixture and will only show a response to a toxicant if the concentration exceeds its tolerance. Consequently, the responses to a binary mixture are additive only if the concentrations of both toxicants are above their respective tolerance thresholds. However, for quantal responses the tolerances to the toxicants in a mixture may vary from one individual to another in a population; therefore, the response of the test animals depends also upon the correlation between the susceptibilities of the individual organisms to the discrete toxicants. For example, in order to predict the proportion of organisms killed by a binary mixture, it is necessary to know not only the proportion that would be killed by each toxicant alone but also to what degree the susceptibility of organisms to one toxicant is correlated with their susceptibility to the other toxicant.

Plackett and Hewlett (1948) recognized this statistical concept and developed mathematical models that accounted for the correlation of individual tolerances ranging from total negative to total positive correlation. If the correlation is completely negative ($r = -1$) so that the organisms most susceptible to one toxicant (A) are least susceptible to the other (B), then the proportion of individuals responding to the the mixture (P_m) can be represented by:

$$P_m = P_A + P_B \quad \text{if} \quad (P_A + P_B \leq 1) \tag{2a}$$

where P_A and P_B are the respective proportion of organisms responding to the individual toxicants A and B. With no correlation ($r = 0$) in susceptibility the relationship is expressed by:

$$P_m = P_A + P_B\,(1 - P_A) \tag{2b}$$

In the limiting case of complete and positive correlation ($r = 1$), individuals very susceptible to toxicant A in comparison with the population will be correspondingly very susceptible to toxicant B. In this situation the proportion of animals responding to the mixture is equal to the response to the most toxic constituent in the mixture. Mathematically this is represented by:

$$\begin{aligned} P_M &= P_A \quad \text{if} \quad P_A \geq P_B \\ P_M &= P_B \quad \text{if} \quad P_B \geq P_A \end{aligned} \tag{2c}$$

For response addition no significance can be placed on the slope of the dose response curves because the toxicants in a mixture are acting primarily upon different biological systems with varying degrees of susceptibility between organisms. Even if the regression equations for the constituents in a mixture are parallel for toxicants acting in this manner, the dose response curve for the mixture will not be linear (Finney 1971). This will be illustrated later for two hypothetical toxicants whose dose response curves are parallel. Although the mathematicl equations (2 a,b,c) representing response addition are relatively simple, the statistical consequences of this type of addition are more complicated than those of concentration addition (Finney 1971).

Terms such as supra- and infra-addition are used to describe toxicant interactions which are greater or less than those predicted on the basis of either concentration or response addition.

Quantal Response Studies

Hypothetical Dose Response Curves

To graphically illustrate the relationship between concentration and response addition, hypothetical dose response curves for two toxicants (A and B) are plotted in Figure 1 expressing percent response in probits as a function of the logarithm of total concentration. In this example the dose response curves for the discrete toxicants are parallel with A being 100 times more toxic than B. We could have also chosen non-parallel curves; however, for these cases equation (1) for concentration addition is not appropriate. Hewlett and Plackett (1959) have developed a more generalized model (from which equation (1) can be deduced) which does not depend on the assumption of parallel dose response curves.

Dose response curves for mixtures of toxicant A and B are obtained when the total concentration is varied and the ratio of the concentrations for the individual toxicants is kept constant. Using the equations (1 and 2 a,b,c) for concentration (C.A.) and response addition (R.A.), dose response curves were calculated for different mixtures containing fixed proportions of toxicants A:B (1:10, 1:100, 1:1000). In Figure 1, the responses to the mixtures are shown graphically in relation to the dose response curves of toxicants A and B.

Several observations can be made from the relationships between the dose response curves in Figure 1. As should be expected, the relative toxicity of the mixture depends on the ratio of its constituents. In Figure 1, a 1:10 mixture is more toxic than the other mixtures depicted because of the greater proportion of the more toxic component - toxicant A. At certain ratios, regardless of the correlation of susceptibility (r), the relative potencies of the mixtures acting in either a concentration or a responsive additive manner are very similar. This is observed in Figure 1 for fixed proportions of 1:10 and 1:1000. Furthermore, for any one ratio the relative potency of the dose response curves for concentration and response addition (r = 1, 0, -1) depends on the level of response. Focussing on the dose response curves for mixtures in the ratio of 1:100, it can be noted that at low levels of response (i.e., at the probit of 2 which corresponds to approximately a 0% response) the mixtures acting in a concentration additive manner are considerably more toxic than those acting by response addition regardless of the degree of correlation (r). This is due to a fundamental difference in the two types of addition. At threshold or below threshold concentrations of toxicants A and B, a mixture acting in a concentration additive manner can elicit a

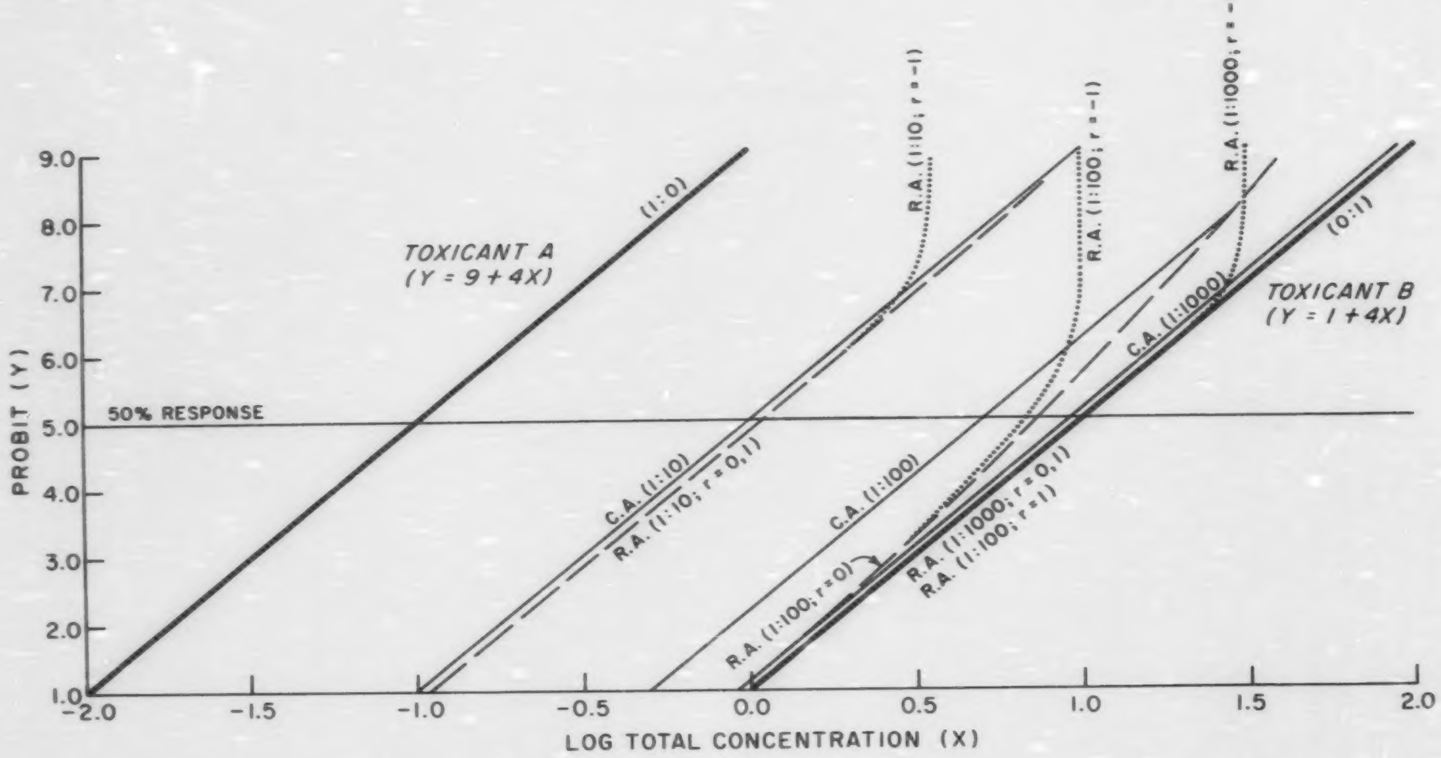

Figure 1. Hypothetical dose response curves for toxicant A (1:0), toxicant B (0:1) and their mixture containing the fixed proportions (1:10, 1:100, 1:1000). See text for explanation.

measurable effect because both toxicants are acting upon similar biological systems. Therefore, their concentrations can sum to produce a concentration for the mixture which is above the threshold level. However, the responses to toxicants acting upon different biological systems (response addition) are only additive if each toxicant in a binary mixture is present in concentrations above their respective threshold levels. For similar reasons, as the concentrations for the toxicants in a 1:100 mixture increase, the dose response curves for response addition (except in the special limiting case where $r = 1$) become progressively more toxic relative to the dose response curve for concentration addition. It is even possible that at high levels of response (in this example, for responses greater than 84% probit of 6.0) mixtures acting in a response additive manner with negative correlation of susceptibility ($r = -1$) can be more toxic than those acting on the basis of concentration addition.

These factors --- the type of interaction, the ratio of the toxicants in a mixture, and the level of response --- must also be considered along with the toxic properties of the individual toxicants in assessing the relative toxicity of a mixture. The failure to recognize these factors can potentially lead to erroneous conclusions concerning the nature of the interaction of multiple toxicants.

Isobole Diagram

It is difficult to visualize the relationships between the dose response curves in Figure 1 primarily due to the number of curves presented. However, the relationships between the hypothetical curves in Figure 1 can be readily conceptualized with isobole diagrams, a technique introduced by Loewe (1928, 1953). Isoboles are lines of equivalent response. They are constructed by plotting on a two-dimensional diagram the concentrations of a binary mixture of toxicants that produce a quantitatively defined response, i.e. a 10%, 50% or 90% lethal response. It should be noted that an isobole diagram can be constructed for any level of response and that the relationship between the isoboles may vary depending upon the response level selected.

The isobole diagram for the 50% level of response of the hypothetical dose response curves in Figure 1 is present in Figure 2. The x and y axes in this diagram represent the concentrations of toxicant B and A respectively. The radiating dashed lines or mixing rays correspond to a series of mixtures (A:B) of fixed proportions. If the 50% response is produced by combinations of the two toxicants represented by points inside the square area, the toxicants are additive. Antagonistic interactions are represented by combinations of concentrations falling outside the square.

The isoboles for concentration and response addition are determined from the concentrations of the two toxicants which correspond to the points of intersection between the 50% response line (Figure 1) and the respective hypothetical dose response curves. These concentrations are plotted in Figure 2 on the appropriate mixing ray. The lines connecting these points define the course of the isobole. Concentration addition is represented by the diagonal isobole. For quantal data, response addition is defined by the

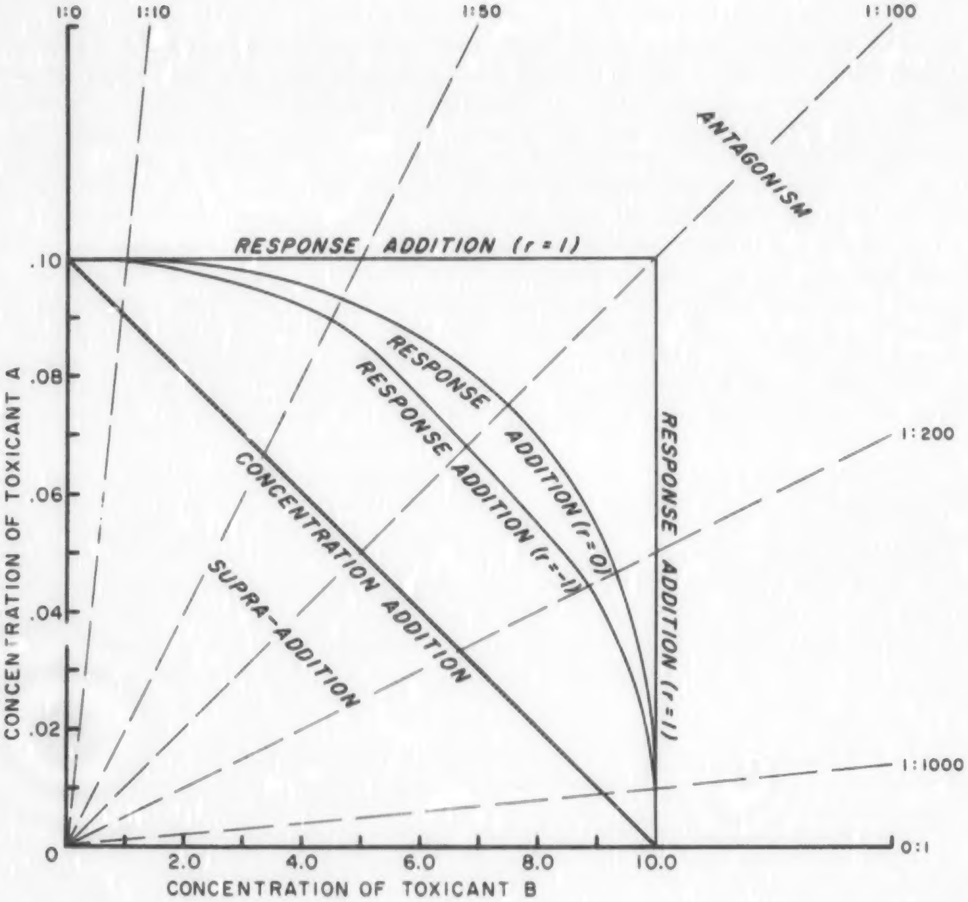

Figure 2. Isobole diagram for quantal response data. Isoboles for concentration and response addition were determined from hypothetical dose response curves in Figure 1.

curved isoboles for complete negative (r = -1) and for no correlation (r = 0) in susceptibility. The upper and right boundaries of the square correspond to the limiting case of response addition with complete positive correlation (r = 1).

The term "no interaction" had been used by other authors (Sprague 1970; Warren 1971) to describe the response additive isobole in Figure 2 corresponding to complete positive correlation of susceptibilities. We recognize that the equation (2c) used to determine this isobole is not additive in a strictly mathematical sense. For example, in lethality studies, organisms whose tolerances to the individual toxicants are positively correlated (r = 1) die in response to the most toxic constituent in the mixture; therefore there is no addition of responses. However, in experimental situations, it is unlikely that complete positive or for that matter complete negative correlation will often be observed. Consequently we have chosen to represent complete positive correlation as a limiting case of response addition to be consistent in our terminology and more importantly to emphasize that the isobole for response addition will for most toxicant mixtures fall between the extreme cases of r = -1 to r = 1 depending upon the degree of correlation.

For reasons similar to the one presented by Warren (1971), we have chosen to use the terms supra- and infra-addition to describe interactions that are greater or less than expected on the basis of either concentration or response addition. It is important that these terms be used in reference to a particular type of addition. For example, an isobole falling between the isoboles for concentration and response addition (r = -1) could be designated as both infra- and supra-additive depending on the nature of the interaction. This potentially confusing situation is avoided by using the terms in the manner we have suggested.

The term antagonism in Figure 2 refers to a physiological or functional antagonism. In the present discussion, we do not consider toxicants which can chemically or physically react in the external medium of an organism to form an inactive or less toxic product (chemical antagonism). Some investigators have used the term antagonism to describe interactions that are less toxic than strict additivity (concentration addition) but whose mixture still has a combined effect greater that either constituent applied alone. We prefer to use the term infra-addition to describe these cases and to reserve antagonism for those cases where the presence of one toxicant necessitates that a higher concentration of another toxicant be present to obtain the defined level of response.

Graded Response Studies

A consideration of the nature of the dose response curves for quantal and graded responses shows that the effects they express are quite different. Quantal dose response curves express the incidence of an all-or-none effect (usually death) when varying concentrations are applied to a group of organisms. The curve is derived by observing the number of organisms which respond or fail to respond at various concentrations. Consequently, the slopes of these curves primarily express the individual variation of the population to a particular toxicant. Graded dose response curves characterize the relationship between the concentration of a toxicant and the magnitude of the effect under consideration. The dose response curve can be derived by measuring on a continuous scale the average response of a group of organisms at each concentration.

As Clark (1937) and others have pointed out, it is possible to represent any graded response as a quantal response provided that the response of each individual organism can be measured. However, this procedure if adopted is at the expense of some "loss of information" (Gaddum 1953). Quantal response data reveals only the number of organisms that respond or fail to respond at some particular concentration. On the other hand, graded response data not only tells us whether or not a group of organisms respond but also how much they respond.

The mathematical equations (2 a,b,c) for the response addition are not appropriate for graded effects for two reasons. First, there is a difference in the way the two types of data are measured. For quantal responses the proportion of organisms responding to any concentration is determined by the ratio of number of organisms showing the response to the total number subjected to the concentration. For graded responses the mean response to each dose is measured but in general the maximum possible response is not known. In cases where the maximal effect is not known, no proportional response can be calculated. This is particularly true for growth experiments where an organism's response can potentially range from growth enhancement to negative growth depending on the concentration of a particular toxicant. Secondly, the statistical concept of correlation between the susceptibilities of the organisms to the discrete toxicants in a mixture is not appropriate for graded responses measured in the manner described earlier. Graded response data represent the average response of a group of organisms. Therefore, the response of each individual organism to the toxicants is not known. To be sure the tolerances of the individuals in the group will vary for the different toxicants in a mixture; however, this factor will not alter the relative toxicity of the mixture because the range of tolerances of the population is theoretically represented in the sample of organisms from this population.

For graded response data, we have represented the combined response to a mixture of toxicants acting in a response additive manner as simply the sum of the intensities of response which each component toxicant produces when administered alone. A similar relationship was defined by Loewe (1953). Concentration addition can be predicted for a toxicant mixture using equation (1) if the component toxicants exhibit parallel dose response curves. Figure 3 represents an isobole diagram for a graded response. The isoboles for concentration and response addition were determined with the appropriate mathematical equations discussed above.

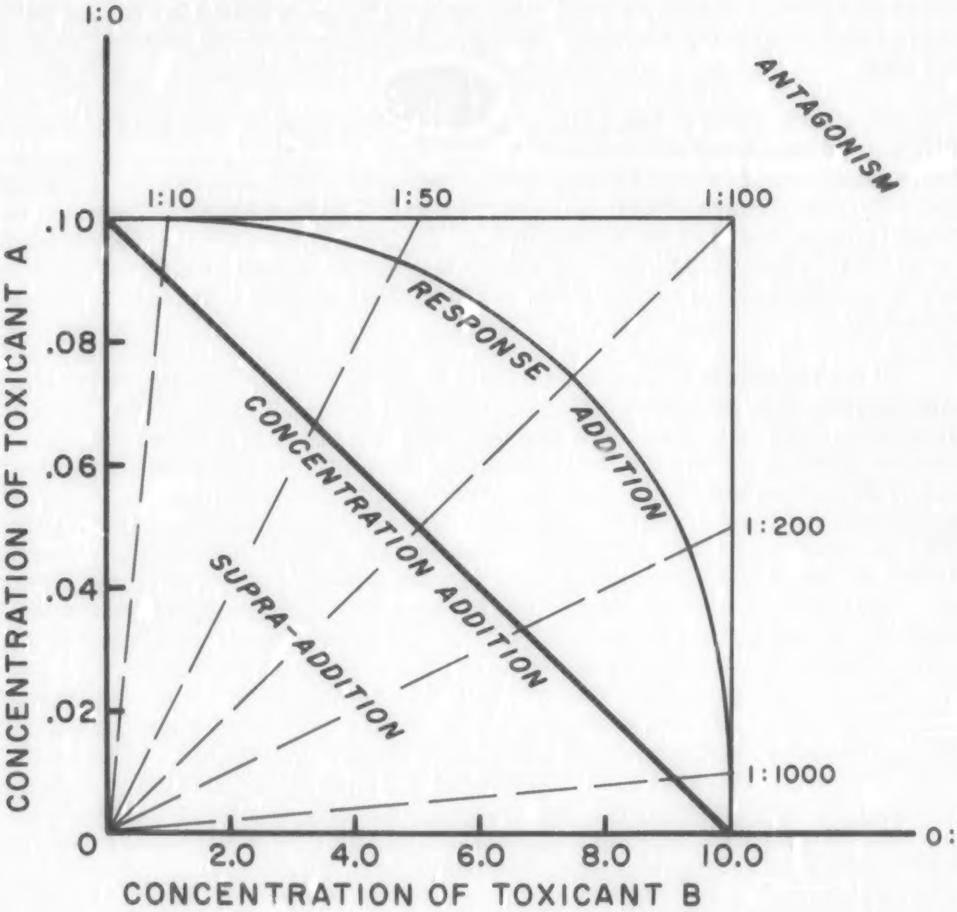

Figure 3. Isobole diagram for graded response data.

The relatively simple types of isoboles represented in Figure 2 and 3 should only be expected for relatively simple in vitro systems or in situations where there is a clear cut relationship between dose and effect. Given the complexity and interdependency of physiological systems, it is reasonable to suppose a priori that the special types of additivity as represented by strict concentration and response addition will be approximated only occassionally in the responses of whole organisms to mixtures of environmental toxicants. Furthermore, as mentioned earlier, the relative toxicity of a mixture depends on several factors which include the level of response (i.e., 10%, 50%, 90% response), the ratio of the toxicants in a mixture (i.e. 1:10, 1:100, 1:1000) and the nature of the response itself. It should be noted that the type of addition can only be described in relation to the response under consideration. With the same mixture of toxicants, different types of toxicant interaction might be expected for different responses (i.e., survival, growth, reproduction). However, these special types of toxicant interaction do provide a frame of reference for evaluating the effects of toxicant mixtures on whole organism performances.

Isobole diagrams are useful for visualizing the relationship between different types of toxicant interactions and for delineating the various factors which can influence the relative toxicity of multiple toxicants. However, in practice, isoboles are difficult to derive requiring a series of dose response curves for the mixture at different ratios of the component toxicants. Furthermore, there is no statistical criteria which might be used to distinguish between one form of interaction and another (Plackett and Hewlett 1952). Following the procedures of Anderson and Weber (1977) we empirically studied the interaction of copper and nickel by deriving a dose response curve for the mixture at one fixed proportion. The dose response curve determined for the mixture was statistically compared to curves predicted on either the basis of concentration or response addition. This approach, utilized by Anderson and Weber (1977) for lethality studies, was adopted in the present study in order to test its applicability to graded response data.

EXPERIMENTAL STUDIES

Lethality Studies

Anderson and Weber (1977) conducted a series of 96 hour bioassays, studying the effects of copper, nickel and their mixture on the survival of male guppies. Statistical tests suggested that the individual dose response curves derived for copper and nickel were parallel. Based upon this observation, it was assumed that the mixture would be concentration additive. To test this prediction, Anderson performed experiments exposing test organisms to a series of mixtures of the two toxicants at a fixed proportion. A statistical comparison of the observed dose response curve to the regression equation calculated on the basis of equation (1) indicated that the assumption of concentration addition adequately described the joint toxicity of the mixture. Using a similar experimental procedure he demonstrated that a mixture of copper and zinc was supra-additive relative to concentration addition. Further studies showed that separate binary mixtures of dieldrin and potassium cyanide and potassium pentachlorophenate and potassium cyanide were response additive.

Growth Studies

Growth was selected as the graded response for this study because it represents a performance of the integrated activities of the whole organism and as such is often a sensitive indicator of the suitability of the environment (Warren 1971). Two of the ways environmental toxicants can affect the growth of an organism are: (1) alter its ability to assimilate and convert food material into body tissue, and/or (2) change its rate of food consumption. To determine the manner in which toxicants affect the growth of an organism, both processes were investigated separately. The methodological and statistical procedures along with the complete results of this study will be published at a later date; however, the results of a preliminary analysis of this data are discussed.

Juvenile guppies were fed daily a restricted ration of tubificid worms to determine the effect of the toxicants on the gross growth efficiency and relative growth rate (as defined by Warren 1971) of the fish. The effect of the individual toxicants and their mixture on food consumption was investigated by feeding groups of fish an unrestricted ration and measuring the amount of worms consumed.

Statistical tests comparing the slopes of the individual dose response curves for copper and nickel derived for each response suggested that they were parallel. On the basis of the mathematical model for concentration addition, equations for the predicted dose response curves were calculated and statistically compared to the regression equations experimentally determined for the mixture. The results indicate that the effects of the toxicant mixture on the gross growth efficiency of the fish subjected to both the restricted and unrestricted feeding regimes are predictable on the basis of concentration addition. However, the dose response curves for the mixture representing the effects of the toxicants on the food consumption of the fish was supra-additive relative to the dose response curve predicted on the basis of concentration addition. Because of the relationship between growth, gross growth efficiency, and food consumption, the effects of the mixture on the relative growth rate are similar to the ones observed for gross growth efficiency at the restricted ration (concentration addition) and for food consumption at the unrestricted ration (supra-addition).

CONCLUSIONS

The results indicate that the assumption of concentration addition adequately predicts the effects of a copper-nickel mixture on both the survival and gross growth efficiency of guppies. The dose response curves for the mixture representing the effects of the toxicants on the food consumption of the fish was supra-additive relative to the dose response curve predicted on the basis of concentration addition. An explanation for the differences in these two responses to the mixture is beyond the scope of the present study.

However, it is possible that the effects of the toxicants on the metabolic processes involved in the conversion of food material into body tissue might be somewhat different than their effects on the biological processes regulating the consumption of food.

In our studies we found that the mathematical model for concentration addition predicted the responses of guppies to both lethal and sublethal concentrations of a copper and nickel mixture. However, it should not be inferred from these results that the type of joint toxicity observed when organisms are subjected to high, rapidly lethal concentrations of mixtures will necessarily occur in cases where animals are subjected to low concentrations of the same toxicants. Furthermore, the nature of toxicant interaction can only be meaningfully described in relation to the particular response under consideration. For example we found that mixtures of copper and nickel were concentration additive in experiments evaluating their effects on the gross growth efficiency of the guppies; however, in the food consumption studies, the same mixture at similar concentrations produced a more toxic response than was predicted on the assumption of concentration addition.

To insure the success of a species in nature, it is necessary to evaluate the effects of potentially hazardous toxicant mixtures on the performances of whole organisms. The proposed approach provides a methodology for assessing the toxicity of mixtures of environmental toxicants at this level of biological organization. However, to offer explanations as to why mixtures of environment toxicants interact in a particular manner requires knowledge of the effects of combined toxicants on underlying biochemical processes and physiogical functions. Such studies will be useful for evaluating the assumptions of the proposed approach and in suggesting other possible types of toxicant interaction.

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RELATIONSHIP BETWEEN pH AND ACUTE TOXICITY OF FREE CYANIDE AND DISSOLVED SULFIDE FORMS TO THE FATHEAD MINNOW

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CREDIT

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INTRODUCTION

There are several fish surfaces where exchange of gases and ions between blood and water can occur, but the gill epithelium is recognized as the primary site. Generally ions have less toxicity than the more lipid-soluble un-ionized molecules. Large hydrated ions are less toxic because of the difficulty in penetrating strongly charged membranes. Ions are repulsed by the charged protein surfaces of the membranes or adsorbed to the membranes. The acute toxicity to fish of solutions containing free cyanide (i.e., HCN plus CN^-) of dissolved sulfide (i.e., H_2S, HS^- and S^{2-}) is mainly attributed to the toxic action of molecular HCN of H_2S. The toxicity is related directly to the concentration of these gases in solution and inversely to pH, and is largely independent of the concentrations of the CN^- or HS^- and S^{2-} anions, which are considerably less toxic than the molecular forms (Doudoroff and Katz 1950; Bonn and Follis 1967; Doudoroff 1976).

Two different gill permeability theories have been proposed to explain the observed changes in toxicity of ammonium salt solutions to aquatic organisms with change of pH. Lloyd and Herbert (1960) suggested that only un-ionized NH_3 is effective and that it is not the pH value of the test solution that is important in determining the toxicity of ammonium salts to fish, but it is the pH value of the solution at the gill surface which controls the concentration of NH_3 at the penetration site. The gill surface pH supposedly depends on the amount of respiratory CO_2 excreted, which lowers the pH value of the solution in contact with the gills. A second theory (Tabata 1962) stated that the ionized fraction of ammonia penetrates membranes and has a measurable toxicity considerably less than the more rapidly penetrating molecular form. Both theories for ammonium salts appear generally applicable to the toxicity of weak bases and weak acids. The Theories presumably can be used to assess the toxicity of other poisons, affected by pH changes within the range tolerated by fish.

When HCN or H_2S gas are dissolved in water, ionization equilibria are established that can be represented by the equations:

$$HCN_{(aq)} \overset{K_a}{\rightleftarrows} H^+ + CN^- \text{ or } H_2S_{(aq)} \overset{K_1}{\rightleftarrows} H^+ + HS^- \overset{K_2}{\rightleftarrows} 2H^+ + S^{2-} \qquad (1)$$

The second equilibrium constant for dissolved sulfide is small in comparison with the first and can be omitted in equilibrium calculations, since the sulfide ion (S^{2-}) is negligible when the test pH is less than about 11. The K_a and K_1 constants are such that in most natural waters molecular HCN or the hydrosulfide ion (HS^-) can be expected to be the predominant free cyanide of dissolved sulfide forms.

The change in tolerance limits for the fathead minnow (*Pimephales promelas* Rafinesque) and dissolved sulfide forms were studied as a function of pH, because the toxicity of weak acids and bases is known to be pH dependent. It was anticipated that experimental results could largely be explained by one of the gill permeability theories.

MATERIALS AND METHODS

TEST WATER AND FISH

The experimental well water used in all bioassays had a total hardness of 220 mg/l as $CaCO_3$. A comprehensive analysis of the water was reported by Smith et al. (1976).

Juvenile fathead minnows were used as test organisms to study the toxicity of solutions containing cyanide or sulfide at various pH values. The fathead minnow was chosen as an experimental organism because it can be cultured and maintained in a laboratory, is handled with ease, and has a wide distribution in chemically diverse natural waters, including those of acid bog lakes and lake waters of high pH. The fathead minnows used in all the bioassays were cultured in the laboratory from a stock originally obtained from the U.S. Environmental Protection Agency's Environmental Research Laboratory in Duluth, Minnesota. The minnows were reared in the laboratory under a constant photoperiod in 30-liter glass aquaria receiving a continuous supply of well water at 25 C and with a pH of approximately 7.9. We believed that the inbred laboratory strain of fish would have a uniform sensitivity to the toxicants not too different from that of other stocks tested at different times, and that possible adverse effects of disease stress and/or treatment could be avoided by not using wild stocks.

Six lots of fish were tested during separate 15-week periods in each of the cyanide and sulfide series. The fish were all approximately 13-weeks old at testing, had mean total lengths of 30.8 and 30.1 mm, and had mean wet weights for survivors of 0.289 and 0.293 g in the cyanide and sulfide bioassays, respectively. The fish were fed Oregon Moist and Glencoe pelleted food twice daily until one day before exposure to the toxicants.

TESTING CONDITIONS

The 96-hr toxicity bioassays were performed in three identical diluter and test chamber units, each including one control and four treatment chambers. The experimental glass chambers measured 50 x 25 x 20 cm high and contained 20 liters of test solution. The intermittent water delivery and toxicant introduction systems were modifications of those described by Brungs and Mount (1970) and Mount and Warner (1965), respectively. Flow through each chamber was at the rate of 500 ml/min, affording 99% replacement in about 3 hours.

The pH of the test water was controlled by dispensing a sulfuric acid or sodium hydroxide solution with a "dipping bird" into the head reservoirs. The temperature of the test water was thermostatically controlled at 20 C. The test water was aerated in the head reservoirs to maintain dissolved oxygen concentrations in the test chambers near 7.5 mg/l. Each test chamber was illuminated for 12 hours each day with a 40-watt incandescent bulb placed 10 inches above the chamber.

Stock solutions of sodium cyanide or sodium sulfide were prepared with reagent grade chemicals and deionized water. One pellet of sodium hydroxide was added to each liter of stock solution to raise the pH, thus retarding escape of HCN or H_2S from the "dipping bird" reservoirs. The solutions were dispensed to the reservoirs from Mariotte bottles. Three days before initiation of the bioassays, 10 or 20 fish acclimated to 20 C for one week were randomly distributed among the 12 treatment and 3 control chambers. Sulfuric acid or sodium hydroxide was then slowly added to the head reservoirs to attain the desired pH. The fish were acclimated to the specified pH for at least two days before introduction of the toxicants.

CHEMICAL ANALYSIS

During each bioassay, water temperature, dissolved oxygen (DO), and pH in each test chamber were measured daily. Alkalinity was determined daily in each control chamber by potentiometric titration with a standard 0.02 N H_2SO_4 solution to the successive bicarbonate and carbonic acid equivalence points, indicated by inflections of the titration curve. Dissolved oxygen was measured with a galvanic-type membrane electrode meter precalibrated by the Winkler method, and pH with a Corning Model 112 glass electrode meter standardized with two primary buffers (APHA 1975). The free carbon dioxide concentrations in test solutions were derived by the nomographic method (APHA 1975) from the pH, temperature, bicarbonate alkalinity, and total filtrable residue of 247 mg/l.

Free cyanide concentrations in each chamber were determined daily by the pyridine-pyrazolone colorimetric method (APHA 1971). Dissolved sulfide concentrations, which were determined to be essentially the same as total sulfide concentrations, were measured in treatment chambers at least twice daily. Water samples taken from the center of each test chamber were stabilized with zinc acetate and analyzed for sulfide by the methylene blue colorimetric procedure (APHA 1971). The concentration of molecular HCN or H_2S was calculated for each free cyanide or dissolved sulfide determination using the daily pH and temperature measurement and the pK equilibrium constants i.e., -log K) calculated by using the equations $pK_{HCN} = 3.658 + 1662/T$ (Broderius, unpublished data) and $pK_{H_2S} = 3.122 + 1132/T$ (Broderius and Smith 1977), where T is temperature in degrees Kelvin. The ratio [HCN]/[free cyanide] or $[H_2]$/[dissolved sulfide] is taken to be equal to $1/(1 + 1p^{pH} - pK_a)$, assuming $[S^{2-}]$ to be negligible. When the molecular HCN or H_2S and free cyanide or dissolved sulfide concentrations are expressed in the same units and as the molecular form, then free cyanide or dissolved sulfide times the appropriate factor (i.e., the ratio computed as shown above) will equal molecular HCN or H_2S.

STATISTICAL ANALYSIS

Estimates of the concentration of cyanide or sulfide most likely to cause 50% mortality (LC50) after 96 hours of exposure were made from lines fitted mathematically by the BMD03S log-probit analysis computer program (Dixon 1973). The data from toxicity tests conducted at a specific pH aim were composited for probit analysis. The 96% confidence intervals for LC50 values were computed according to formulas proposed by Litchfield and Wilcoxon (1949) and Finney (1971). $(Chi)^2$ tests were applied to each group of data to determine variability and acceptability. When heterogeneous data were indicated, the appropriate adjustment in the 95% confidence intervals for LC50 values were made.

RESULTS AND DISCUSSION

The relationship between test pH and acute toxicity of free cyanide and dissolved sulfide forms to the fathead minnow at 20 C was determined for pH values ranging from about 6.8 to 9.3 and 6.5 to 8.7, respectively. The test conditions and log-probit analysis of composite test results grouped according to pH are summarized in Table 1-3 and Figure 1. It is apparent that the 96-hour median lethal concentrations (LC50) of free cyanide and molecular HCN were little different and fairly constant within the pH range 6.8 to 8.3. Beyond this pH to pH 9.3, the values diverged markedly, with the free cyanide values increasing and the HCN values decreasing.

Except for some increase with rise of pH from about 6.5 to about 7.1

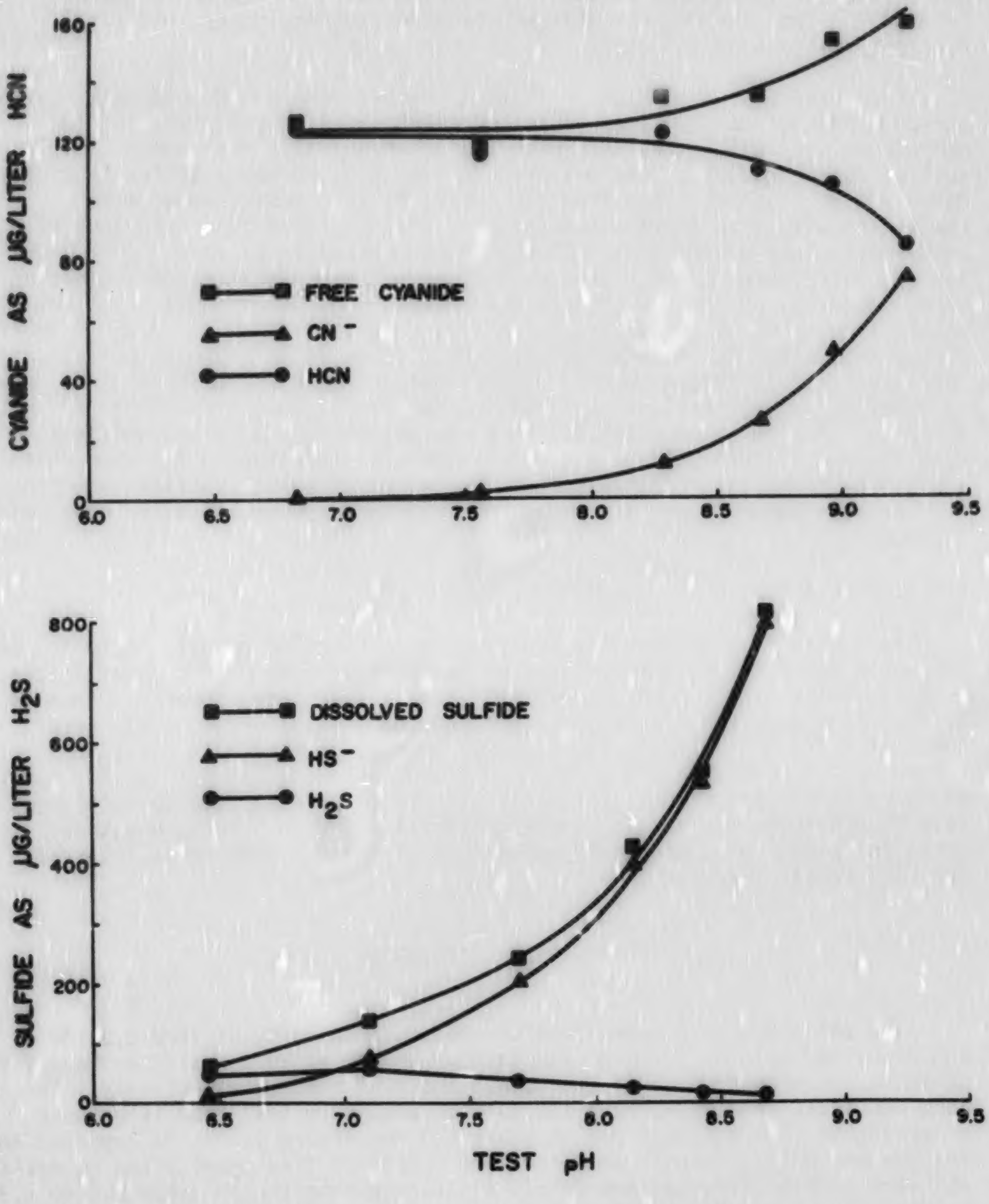

Figure 1. Relationship between test pH and 96-hr LC50 cyanide or sulfide concentrations for the fathead minnow at 20° C.

Table 1. Mean test conditions for composite free cyanide and dissolved sulfide bioassays: standard deviations in parentheses

			Alkalinity, mg/l $CaCO_3$		Free CO_2 in test solution*	
Test pH	Temperature, C	DO. mg/l	Bicarbonate	Total	mg/l	mm Hg
			Cyanide Series			
6.830(0.028)	20.1(0.12)	7.65(0.06)	96	96	28.0	12.4
7.559(0.041)	20.0(0.02)	7.56(0.09)	193	193	11.0	4.9
8.286(0.034)	20.0(0.01)	7.66(0.06)	240	240	2.5	1.1
8.672(0.067)	20.1(0.09)	7.59(0.09)	229	244	1.0	0.44
8.974(0.068)	20.0(0.12)	7.71(0.10)	214	243	0.47	0.21
9.262(0.091)	20.2(0.09)	7.63(0.12)	206	250	0.24	0.11
			Sulfide Series			
6.462(0.070)	20.0(0.05)	7.62(0.08)	63	63	44.0	19.5
7.101(0.030)	20.0(0.04)	7.66(0.09)	128	128	20.5	9.1
7.698(0.036)	20.0(0.20)	7.65(0.07)	198	198	7.9	3.5
8.151(0.030)	20.0(0.11)	7.49(0.24)	229	229	3.2	1.4
8.430(0.018)	20.0(0.05)	7.55(0.11)	234	238	1.7	0.75
8.693(0.014)	20.1(0.10)	7.49(0.11)	234	243	1.0	0.44

* Free CO_2 evaluated by nomographic method (APHA 1975). Assuming $K = H_2CO_3/P_{CO_2}$ and $\log K = -1.41$ at 20 C and one atmosphere, then 1 mg/liter CO_2 = 0.444 mm Hg CO_2 tension (Stumm and Morgan, p. 148, 1970).

Table 2. Analysis by the log-probit method of the results of fathead minnow bioassays of cyanide at 20 C, with tests grouped according to pH

	Log-probit regression analysis*				95% confidence
Test pH	Treatments	α	β	96-hr LC50, µg/l as HCN	limits for LC50, µg/l
			HCN		
6.830	6	-23.80	13.76	124	106 - 144
7.559	7	-23.47	13.81	115	102 - 130
8.286	6	-27.37	15.52	122	115 - 128
8.672	9	-33.46	18.88	109	105 - 113
8.974	17	-16.52	10.68	104	96 - 112
9.262	10	-28.73	17.55	83	80 - 87
			Free Cyanide		
6.830	6	-23.82	13.76	124	107 - 144
7.559	7	-23.60	13.82	117	102 - 135
8.286	6	-28.00	15.54	133	126 - 140
8.672	9	-34.59	18.64	133	128 - 138
8.974	17	-16.44	9.83	152	140 - 165
9.262	10	-28.57	15.29	157	142 - 173

* For equation $Y_i = \alpha + \beta (\log X_i)$ when Y_i is the maximum likelihood probit value and X_i is log cyanide concentration as µg/l HCN (Dixon 1973).

Table 3. Analysis by the log-probit method of the results of fathead minnow bioassays of sulfide at 20 C, with tests grouped according to pH

	Log-probit regression analysis*				
Test pH	Treatment	α	β	96-hr LC50, µg/l as H_2S	95% confidence limits for LC50, µg/l
			H_2S		
6.462	11	- 9.85	8.78	49.2	45.1 - 53.7
7.101	8	-14.12	10.88	57.2	52.6 - 62.1
7.698	8	-18.26	14.70	38.2	32.8 - 44.5
8.151	6	-31.70	25.98	25.8	24.7 - 27.0
8.430	6	-30.67	28.19	18.4	17.5 - 19.4
8.693	9	-11.07	13.68	14.9	13.0 - 17.2
			Dissolved Sulfide		
6.462	11	-10.64	8.65	64.1	58.7 - 70.0
7.101	8	-18.57	11.09	133	123 - 145
7.698	8	-39.54	18.73	239	219 - 260
8.151	6	-52.98	22.10	420	394 - 448
8.430	6	-52.31	20.94	546	483 - 616
8.693	9	-40.23	15.56	806	726 - 895

* For equation $Y_i = \alpha + \beta (\log X_i)$ when Y_i is the maximum likelihood probit value and X_i is log sulfide concentration as µg/l H_2S (Dixon 1973).

the 96-hr LC50 concentrations of molecular H_2S decreased as the test pH increased. These values, in μg/liter H_2S ranged from 57.2 at pH 7.1 to 14.9 at pH 8.7. Within this pH range, a 0.1 unit increase in pH was calculated by linear regression (r of -0.994) to result in a 2.7 μg/liter decrease in the 96-hr LC50 value of molecular H_2S. However, as pH increased, the concentration of dissolved sulfide in equally toxic solutions increased logarithmically.

The anomalous result for the sulfide experiments performed at a pH of about 6.5 may be due to an interaction between sulfide and the relatively high CO_2 concentration in the test solutions. It is also possible that the LC50 values for H_2S are relatively constant over the pH range of about 6.5 to 7.1 and at a constant free CO_2 level.

LLOYD AND HERBERT'S THEORY

According to the theory of Lloyd and Herbert (1960), the toxicity of cyanide and sulfide solutions to the fathead minnow is increased by depression of pH at the gill surface due to respiratory excretion of CO_2. This change in toxicity results from the conversion of CN^- of HS^- anions to molecular HCN or H_2S in the solution in contact with the gills. The magnitude of this effect depends upon the concentration of free CO_2 present in solution and the shifting of the CO_2-bicarbonate-carbonate chemical equilibrium. When the concentration of free CO_2 in the water is very low (high pH), the addition of respiratory CO_2 considerably reduces the pH value at the gill surface. As the level of free CO_2 rises in the bulk of the solution, the pH change becomes less. The pH at the gill surface can, theoretically, be calculated from the bicarbonate alkalinity, temperature, free CO_2 concentration in the test solution, and the free CO_2 excreted by the gills of the fish by use of the standard nomographic method (APHA 1975). The increase in concentration of excreted CO_2 in the respiratory water (as mg CO_2/liter) was estimated by Lloyd and Herbert (1960) by means of the equation:

$$\text{Increase in } CO_2 = DO \times RO \times \frac{\text{mol. wt. } CO_2}{\text{mol. wt. } O_2} \times 100 \qquad (2)$$

where DO = dissolved oxygen concentration of water in mg/liter

RQ = respiratory quotient of the fish

P = percentage of oxygen removed from the respiratory water by the fish

Kutty (1968) has determined that the respiratory quotient (RQ) is essentially unity when freshwater fish are spontaneously active in nearly air-saturated water. Since the CO_2 is excreted along the surface of the lamellae, a pH gradient may be present in the gills. Lloyd and Herbert proposed that the average pH be taken to be that which is produced when half of the total amount of CO_2 excreted is at equilibrium with the carbonate system. An analysis of the cyanide and sulfide bioassay data by their procedure is summarized in Table 4. The apparent change in HCN and H_2S toxicity with change of test pH could mathematically best be reconciled with this theory by assuming a respiratory quotient of 1.0 and that the fathead minnow removed from the respiratory water about 10 and 55% of the dissolved oxygen in the cyanide and sulfide bioassays, respectively. If a respiratory quotient of less than 1.0 is assumed, as proposed by Lloyd and Herbert (1960), then a greater percentage of the dissolved oxygen available at the gills would need to be removed for conformance with the theory.

The above proposed explanation of the relation to pH of the toxicity to fish of weak acids and bases is interesting, but for a number of reasons it may not be appropriate. First, it was assumed by Lloyd and Herbert in their calculations that the utilization of oxygen initially present in the water passing over the gills of rainbow trout is about 80%. Recent studies have shown that the percentage of oxygen removed is variable among individuals of the same and different species and for a given fish under different conditions and at different times. According to a review by Shelton (1970), almost all of the reported utilization values are less than 80% under nearly ideal environmental conditions and the utilization is usually less than 50% when there is hypoxic stress (Holeton and Randall 1967; Davis and Cameron 1971). In view of the stress occurring in an acute toxicity bioassay, it is not unreasonable to assume that the utilization might even be considerably less than the 55% value necessary for agreement with the pH drop at the gill theory in the case of the sulfide bioassays. In attempting to explain the 96-hr LC50 bioassay results (Table 4) obtained in tests performed under nearly identical environmental conditions, the percentage utilization of dissolved oxygen at the gills must be 5.5 times as great for fathead minnows tested in sulfide solutions as for those tested in the cyanide solutions. This discrepancy is an additional reason why the theory is not viewed as an appropriate explanation of the relation to pH of the toxicity of weak acids to fish.

The respiratory quotient of fish may not remain constant throughout the duration of an acute toxicity test. According to Kutty (1968, 1972) and Kutty et al. (1971), the *in vivo* RQ can increase upon marked reduction of DO as a result of the accumulation of lactic acid in the tissues and consequent release of CO_2 from the bicarbonate reserve, which take place when metabolism is partially anaerobic. Because of the nature of the toxic action of cyanide and sulfide, a similar response may be expected of fish dying from these poisons. If the RQ does increase, the percentage of available oxygen removed at the gills could decrease and still be accompanied by an increase in CO_2 at the gills.

A second weakness of the theory of Lloyd and Herbert is apparent when one examines the manner and amount in which CO_2 is excreted at the gills. According to Dejours et al. (1968), when the respiratory quotient is near unity, the changes in CO_2 tension of the water passing over the gills of teleost fish are small. In their experiments with goldfish at 25 C, this change was less than 1.0 mm Hg. The change in tension of highly soluble CO_2 in well-aerated, high-carbonate water is small because of the high rate of ventilation which is necessary to extract poorly soluble oxygen. The

Table 4. Average estimated 96-hr LC50 concentrations of molecular HCN or H_2S at the fathead minnow gill surface assuming a dissolved oxygen utilization of 10 and 55 percent for the cyanide and sulfide bioassays at 20 C, respectively, and an RQ of 1.0.

Test pH	Average free CO_2 at gill,[1] mg/l	Average pH at gill[2]	One-half pH decrease at gill	Ionization factor for gill pH[3]	Free cyanide or dissolved sulfide 96-hr LC50, µg/l	HCN or H_2S at gill to give 96-hr LC50, µg/l
			Cyanide Series			
6.830	28.53	6.82	0.01	0.997	124	124
7.559	11.52	7.52	0.04	0.985	117	115
8.286	3.03	8.19	0.10	0.932	133	124
8.672	1.52	8.50	0.17	0.871	133	116
8.974	1.00	8.68	0.29	0.816	152	124
9.262	0.76	8.81	0.45	0.767	157	120
						Mean 120
						SD 4.2
			Sulfide Series			
6.462	46.88	6.41	0.05	0.789	64	50.5
7.101	23.40	7.04	0.06	0.467	133	62.3
7.698	10.79	7.57	0.13	0.205	239	49.0
8.151	6.13	7.87	0.28	0.115	420	48.2
8.430	4.55	8.01	0.42	0.0858	546	46.8
8.693	3.83	8.10	0.59	0.0709	806	57.1
						Mean 52.3
						SD 6.1

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[1] Free CO_2 in test solution (Table 1) plus one-half increase in CO_2 at gill surface (Lloyd and Herbert 1960).

[2] Calculated from average free CO_2 at gill, using nomograph for evaluation of CO_2 (APHA 1975).

[3] Factor = $1/(1 + 10^{pH - pK_a})$ when pK_{HCN} + 9.328 and $pK_{H2S} = 6.983$ at 20 C.

carbonate-bicarbonate system absorbs a considerable amount of CO_2 produced by respiring fish, and the titration alkalinity increases as a result of NH_4^+ excretion in conjunction with active ion exchange for Na^+ (Dejours et al., 1968). Holeton and Randall (1967) determined that the P_{CO_2} of the blood in the vental aorta was greater by about 1.0 mm Hg than that in the dorsal aorta of rainbow trout both in well-aerated water and in a hypoxic environment. Rahn (1966) stated that the change in CO_2 tension of the water at the gills could not be much greater than 5 mm Hg at 20 C, and it could be that great only if nearly all the O_2 in the water passing over the gills was extracted.

The mechanism for the excretion of metabolic CO_2 by freshwater fish includes the catalytic conversion to bicarbonate of some of the CO_2 in the blood by carbonic anhydrase in the gill epithelium (Randall 1970). Therefore, along with the free CO_2 entering the water, bicarbonate passes across the gill epithelium by an active exchange mechanism which involves chloride. Stumm and Morgan (1970) indicated that the hydration/dehydration reaction of $CO_{2(aq)} + H_2O \rightleftarrows H_2CO_3$ proceeds slowly in water, the establishment of the hydration equilibrium at pH values near 7 requiring a finite time on the order of many seconds. The formation of CO_2 from the bicarbonate actively excreted by the gill epithelium is also slow. Water is generally considered to pass over the gill epithelium in less than two seconds, and the hydration of CO_2 and formation of CO_2 from bicarbonate in water is on the order of many seconds. Therefore, the major portion of the rise in P_{CO_2} and ultimate pH shift at equilibrium should occur after the water has left the respiratory surface.

To explain the cyanide and sulfide toxicity bioassay data by the theory of Lloyd and Herbert, the total increases in CO_2 at the gill surface when oxygen utilization is 10 and 55%, respectively, would need to average about 1.0 and 5.7 mg/l or 0.44 and 2.5 mm Hg, respectively. These increases, although physiologically possible, are greater for the sulfide bioassays than those that have been reported in the literature (Randall 1970). The accompanying maximum total pH change necessary for agreement with the theory would need to be 0.9 and 1.2 units for the cyanide and sulfide bioassays at the highest test pHs, respectively. These changes appear to be extreme, since Holeton and Randall (1967) measured a 0.2 pH unit difference between water samples from the buccal and opercular chambers of the rainbow trout. Because of all the above considerations, it is concluded that the theory of Lloyd and Herbert is not an appropriate explanation of the relation of toxicity to pH observed in the reported cyanide and sulfide bioassays.

TABATA'S THEORY

It is apparent from examination of the data in Tables 2 and 3 and Figure 1 that with an increase in test pH more free cyanide or dissolved sulfide becomes necessary to produce the acute response. However, the increase in concentration needed is not large enough to maintain a constant 96-hr LC50 concentration of molecular HCN or H_2S. If the toxicity of free cyanide and dissolved sulfide is attributable to the molecular component only, the slopes of curves relating the proportion of weak acid present in the molecular form and the toxicity to test pH should be parallel.

Inspection of these relationships plotted in Figure 2 shows that this is not the case. The discrepancy between the curves occurs mainly in the alkaline region where the ratio of weak acid anions to total acid increases rapidly with rise of pH. Therefore, it appears that the CN^- and HS^- anions may have a toxicity equal to at least a fraction of the toxicity of the neutral molecules. The theory of Tabata (1962), which assumed that not only the molecular forms but also the ionized fraction can penetrate membranes and have a measurable toxicity, may thus be appropriate for explaining the toxicity of cyanide and sulfide solutions to fish.

The relationship expressing Tabata's theory where the total toxicity is equal to the sum of the toxicities due to the molecular and ionic forms can be represented by the equation:

$$\frac{C}{LC50} = T_m \text{ [molecular form] } + T_i \text{ [ionic form]} \qquad (3)$$

where C is the total concentration of molecular plus ionic forms, 1/LC50 is an expression of total toxicity, and T_m and T_i are the molar toxicities of the molecular and ionic forms, respectively. The ratio between the toxicity of the molecular and ionic forms (T_m/T_i) can be derived from the LC50 determined at one pH and that (LC50') determined at another pH (pH') in the manner shown in Appendix A. This relationship for weak acids was defined by Tabata as follows:

$$\frac{T_m}{T_i} = \frac{\frac{K}{[H^+]}\left[1 + \frac{K}{[H^+]}\right] LC50' - \frac{K}{[H^+]}\left[1 + \frac{K}{[H^+]'} \quad LC50\right]}{\left[1 + \frac{K}{[H^+]'}\right] LC50 - \left[1 + \frac{K}{[H^+]}\right] LC50'} \qquad (4)$$

where K is the acidic ionization constant. This equation was used to analyze the cyanide and sulfide bioassay data; the calculated T_m/T_i values are presented in Table 5. It was anticipated that the ratios would be fairly constant, that is, independent of the pH. This relationship was essentially the case for the cyanide bioassays, the overall mean ratio being 2.3. The ratios calculated for the sulfide bioassays were somewhat variable but fairly constant when the pH values were between 7.7 and 8.4. The calculated T_m/T_i values within this pH range average about 15, when the value 6.49 is omitted. Therefore, the effective toxicity to the fathead minnow of the HCN and H_2S molecules in solution is apparently about 2.3 and 15 times that of CN^- and HS^- anions, respectively.

The above equation (4) can be modified to estimate a new LC50' for a new pH' from the given pH, LC50, and T_m/T_i values (Appendix A). This relationship for weak acids was defined by Tabata as follows:

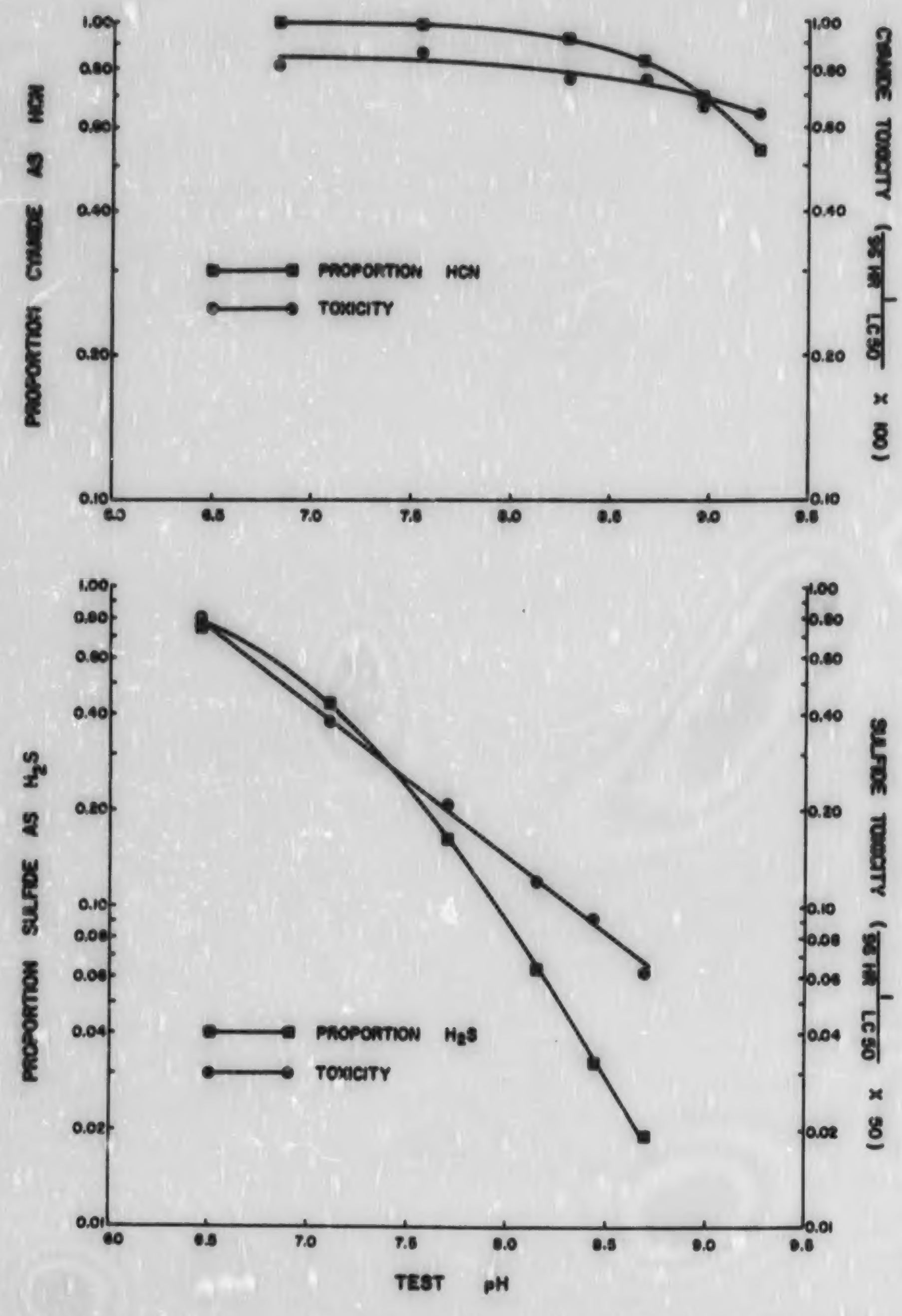

Figure 2. Toxicity of free cyanide or dissolved sulfide to the fathead minnow and the proportion of each present in the molecular form (as HCN or H_2S) in relation to test pH at 20° C.

Table 5. Calculated T_m/T_i ratios and predicted LC50 values for cyanide and sulfide bioassays at different levels of pH

Test no.	pH	T_m/T_i ratio for test number[1]					Free cyanide or dissolved sulfide 96-hr LC50, µg/l[2]	
		1	2	3	4	5	Determined	Predicted
				Cyanide Series				
1	6.830	--	--	--	--	--	124	120
2	7.559	0.18	--	--	--	--	117	120
3	8.286	5.75	-1.40	--	--	--	133	125
4	8.672	1.60	3.45	1.01	--	--	133	133
5	8.974	2.51	4.42	2.14	6.08	--	152	145
6	9.262	1.82	2.26	1.63	1.95	1.23	157	162
				Sulfide Series				
1	6.462	--	--	--	--	--	64	66
2	7.101	-7.22	--	--	--	--	133	111
3	7.698	17.3	6.49	--	--	--	239	239
4	8.151	16.8	10.2	16.3	--	--	420	412
5	8.430	16.8	11.6	16.6	17.0	--	546	526
6	8.693	22.9	16.9	25.5	35.2	77.3	806	615

---

[1] Ratio applies to comparison of the test indicated by the test number below with the test at a higher pH whose test number appears in the first column (equation 4). The K_{HCN} values at 20 C are 4.700×10^{-10} and 1.041×10^{-7}, respectively.

[2] Values based on T_m/T_i of 2.3 or 15, and mean of tests 1 and 2 or test 3 for cyanide and sulfide bioassays, respectively.

$$LC50' = \frac{\left[1 + \frac{K}{[H^+]'}\right]\left[\frac{T_m}{T_i} + \frac{K}{[H^+]}\right]}{\left[1 + \frac{K}{[H^+]}\right]\left[\frac{T_m}{T_i} + \frac{K}{[H^+]'}\right]} \cdot LC50 \qquad (5)$$

The predicted LC50' values (Table 5) for the cyanide bioassays are estimates based on the mean pH and LC50 values for tests 1 and 2 and a T_m/T_i ratio of 2.3. The predicted LC50' values for the sulfide bioassays are estimates based on the LC50 experimentally determined at pH 7.7 and a T_m/T_i of 15. As seen in Table 5 and Figure 3, the predicted and determined 96-hr LC50 values generally show good agreement, but at the highest pH value of the sulfide series (pH 8.7) the determined value for dissolved sulfide is decidedly larger than the predicted one.

The percentages of total toxicity attributable at different pH values to the molecular and ionic forms can be calculated from the expressions $[P_m/\{P_m + P_i(T_i/T_m)\}]$ [100] and $[P_i/\{P_i + P_m(T_m/T_i)\}]$[100]respectively, where P_m and P_i represent the proportions of free cyanide or dissolved sulfide in the molecular and ionic forms, respectively. These toxicity relationships, as presented in Figure 4, were calculated assuming T_m/T_i ratios for the cyanide and sulfide bioassays of 2.3 and 15, respectively. The anions contribute a larger proportion of the total toxicity with increasing pH. At pH levels below 9.5, the contribution of the HS^- ion is greater than that of CN^-, even though CN^- is not as much less toxic than HCN as the HS^- ion is less toxic than H_2S. An appreciable deviation from the theoretical relationship, indicated by line A in Figure 4, was noted only in the sulfide bioassay series when the pH was relatively high.

In order to explain more fully the variable relation between the toxicity of solutions of the weak acids and the concentrations of the molecular forms, one should consider the concentrations and forms of the toxicants not only in the test solutions but also in the body fluids. Carbon dioxide is known to diffuse readily through tissues, and the CO_2 tension (P_{CO_2}) in blood vessels efferent to the gills of fishes approximates that of the water in the buccal cavity (Stevens and Randall 1967). An increase in CO_2 content of the water thus produces an increased CO_2 content of the blood. Plasma proteins and hemoglobin buffer the H^+ deriving from the dissociation of carbonic acid, which is formed when CO_2 is hydrated. The buffering capacity is limited; and, as Albers (1970) has stated, the linear relationship between fish blood pH and log P_{CO_2} varies in slope, depending on the buffering capacity. It can be determined from Figure 11 of his review that, in the carp (*Cyprinus carpio*), as the blood P_{CO_2} is increased from about 2 to 12 mm Hg, the pH decreased from about 7.9 to 7.4. Ferguson and Black (1941) determined that, at 15 C, as the blood P_{CO_2} of the carp was increased from 2 to 20 mm Hg, the plasma pH of oxygenated blood decreased from 7.91 to 7.23. A comparable decrease for the rainbow trout was from 7.66 to 7.15. In a study by Hunn (1972) on the effect of thanite on the blood chemistry of the carp, a decrease in O_2 and glucose utilization and accumulation of lactic acid resulting from blockage of the electron transfer chain by cyanide

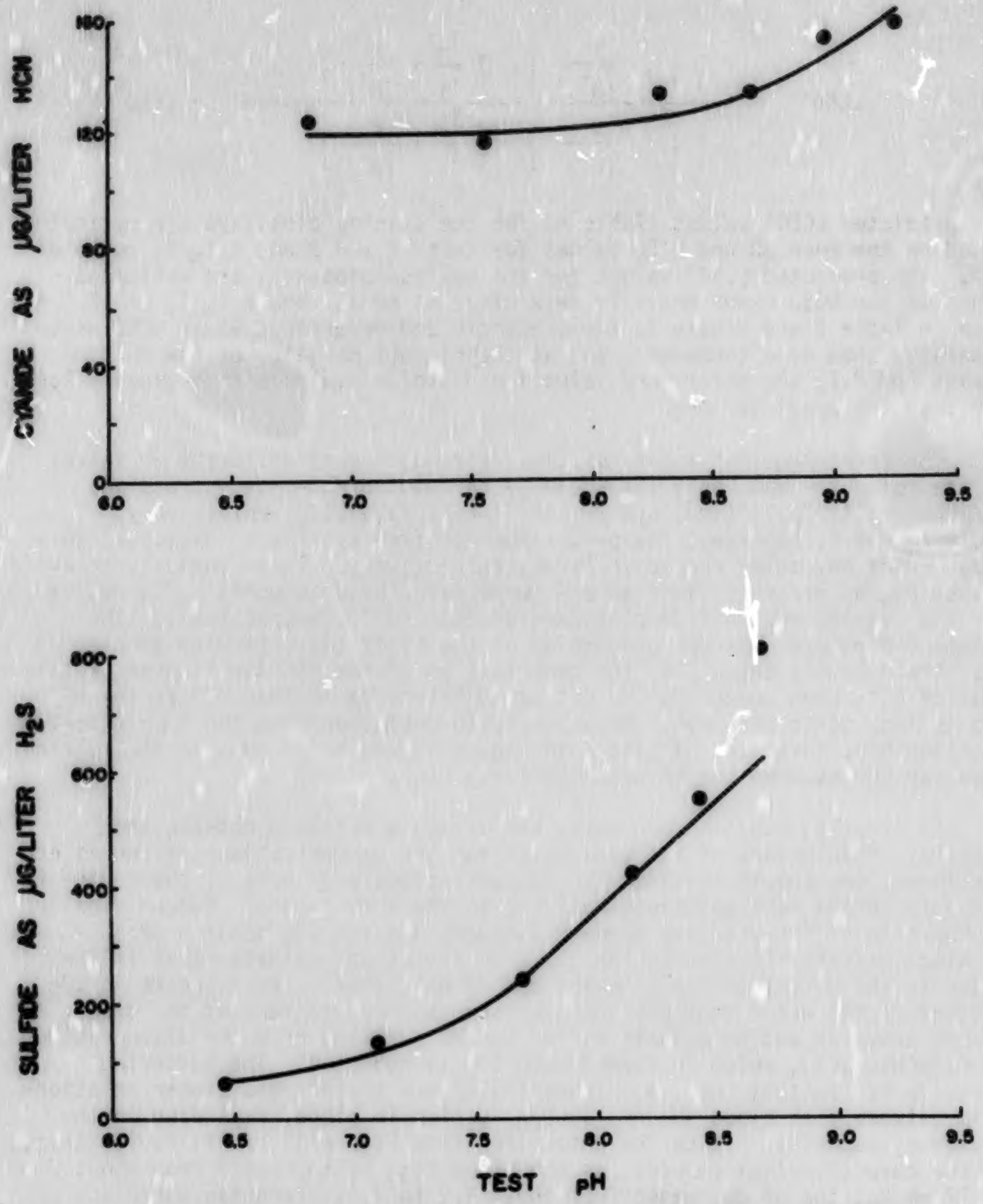

Figure 3. Determined and predicted 96-hr LC50 concentrations of free cyanide and dissolved sulfide for fathead minnow bioassays at different levels of pH. The plotted points represent determined values; the curves were fitted to (connect) the predicted levels.

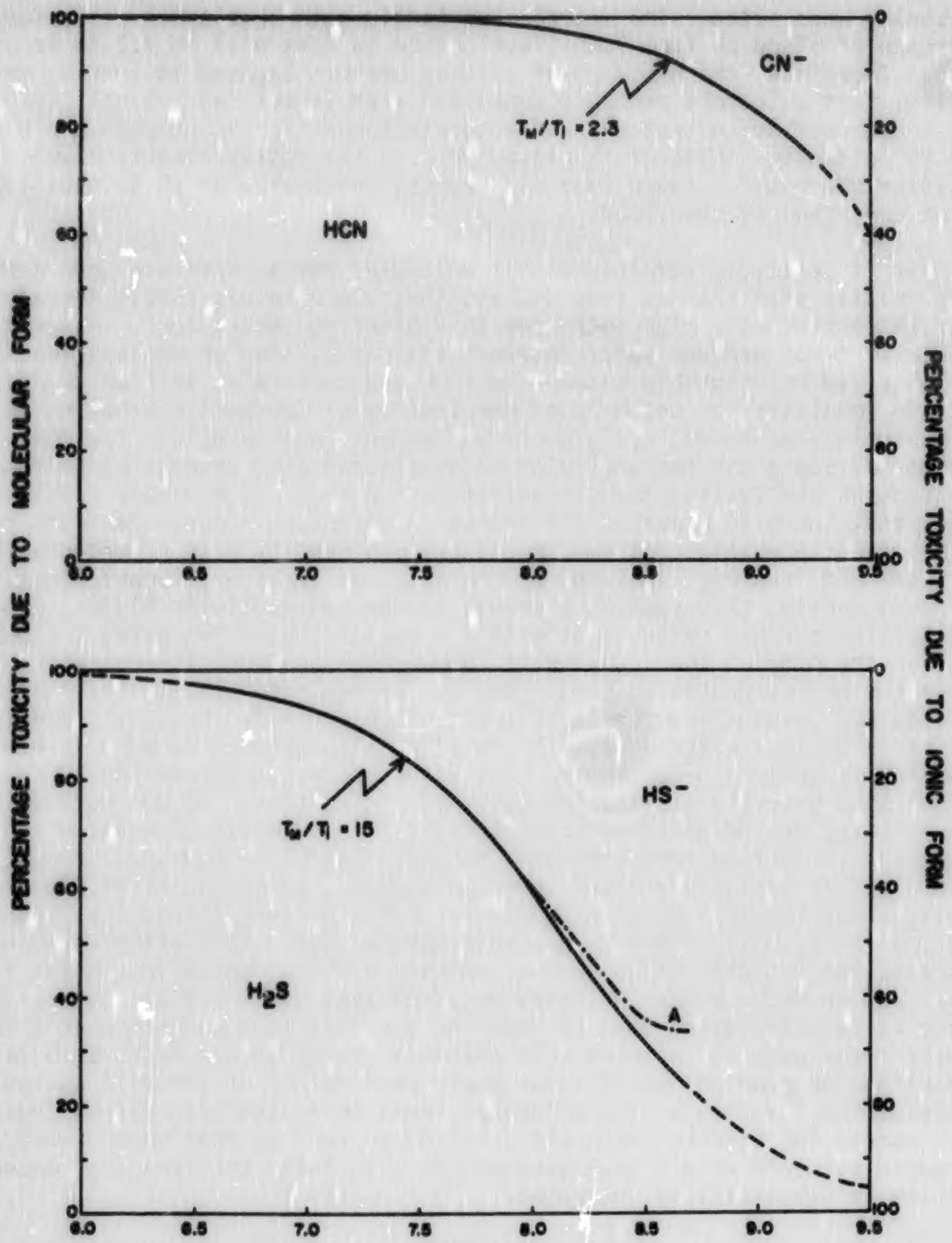

Figure 4. Relations between pH and the percentages of total toxicity attributable to the molecular and ionic forms of cyanide and sulfide in bioassays with the fathead minnow at 20° C. Deviation of experimental data from the theoretical relationship is indicated by Line A.

poisoning were noted. The increase of lactic acid levels was reflected in a decrease of blood pH from 7.85 (level found in controls) to 7.2 in exposed fish. Therefore, the blood pH of fathead minnows exposed to cyanide and sulfide test solutions probably decreased with increasing ambient CO_2 tensions, decreasing test pH, and accumulation of lactic acid in the blood due to poisoning. Changes in plasma pH parallel fairly closely changes in cellular pH, and, if there is a difference, intracellular pH is usually lower than that of the blood.

It is generally recognized that molecular forms penetrate membranes more readily than charged ions do, and that blood levels should increase in fish concurrently with increases in ambient concentrations. Assuming the molecular forms are the major internal toxicants, then an explanation for the observed relationship between test pH and cyanide or sulfide toxicity must include consideration not only of penetration of the gill epithelium mainly by the molecular forms, but also of variations in internal ionization with changes of blood and intracellular pH associated with changes of internal CO_2 tension and lactic acid concentration. Warren and Schenker (1962) proposed that there is a marked difference in effect on ammonia toxicity to mice between equivalent plasma pH changes produced by either strong acids or bases and free CO_2. Infused strong acids or bases will penetrate tissue barriers poorly, thus causing a change in the extracellular fluid-intracellular fluid pH gradient with the redistributed NH_3 being trapped as NH_4^+ on the side of lower pH. With CO_2, the pH gradient is less marked since the pH is changed on both sides of the membrane almost simultaneously because CO_2 crosses membranes with ease. Consequently, NH_3 will tend to redistribute less extensively. The distribution between ammonia levels in fish and in their aqueous environment was proposed by Warren and Schenker (1962) as a possible explanation of Lloyd and Herbert's (1960) data which demonstrated an increased apparent toxicity of un-ionized ammonia with reduction in test pH from increased ambient free CO_2. However, because fish blood is buffered against large pH changes, at high ambient CO_2 tensions and low pH a gradient would favor NH_3 diffusing from the blood into and trapped as NH_4^+ in the more acidic test medium. This effect should decrease the toxicity of un-ionized ammonia with increases in ambient free CO_2. Warren and Schenker's theory does not adequately explain our results, since it is anticipated that by lowering the test pH with strong acid the apparent toxicity of the weak acid molecular forms should increase because the diffusion gradient would favor their penetration of the gill epithelium. Instead, the toxicity of the molecular forms decreased with decreasing test pH. Warren and Schenker apparently failed to realize that when strong acid is added to water with a high bicarbonate alkalinity the free CO_2 concentration is substantially increased.

A change in the permeability of the gills to molecular HCN or H_2S may also contribute to the apparent change in toxicity, but an extent of this change sufficient to entirely account for the decrease in molecular form LC50 values, especially in the sulfide series of tests, is most unlikely.

Cyanide and sulfide each has a specific inhibitory effect upon certain enzymatic processes. According to Hewitt and Nicholas (1963), cyanide may react with metal enzymes as HCN or as CN^-, but the general consensus is that various types of inhibition mainly involve the HCN molecule. Sulfide, including the anionic species, can inhibit certain enzymes by formation of complexes with essential metals contained in the enzymes. Since the molecular forms can move across internal tissue barriers more readily than charged ions do, it seems reasonable to suppose that the effectiveness of cyanide or of sulfide as an internal poison becomes greater with an increase in the proportion existing in the molecular form. Changes in blood pH will thus affect the toxicity by changing the concentration of the freely penetrating molecular form.

If the CN^- and HS^- anions penetrate the gills along with the molecular forms, the toxicity of cyanide and sulfide solutions to fish should not be entirely determined by the ambient molecular levels. Internal conditions should also be taken into consideration. At the pH of blood of the fish in all the cyanide test solutions, high percentages of the free cyanide which penetrated the gills must have existed as HCN. Thus, when the calculated T_m/T_i ratio of 2.3 was used, the predicted 96-hr LC50 values for free cyanide bioassays showed good agreement with the determined values (Table 5). For sulfide, predicted values based on a T_m/T_i ratio of 15 and tests at pH 7.7, at which the blood pH is supposed to be near that of the test solution, good agreement between determined and predicted 96-hr LC50 values was observed, except at pH greater than 8.4. The apparent anomaly may be explained as having been due to the pH of the blood having been slightly higher in fish tested in the more alkaline solutions than in those tested at pH 7.7 and 7.9 mg/l free CO_2. In the bioassays at high pH, more of the sulfide presumably had to penetrate, largely as HS^- ion, in order to produce a toxic effect equal to that produced at pH 7.7. Therefore, a greater amount of dissolved sulfide than predicted was required to produce the toxic response at the high pH. At a high enough pH, the presence of an internal H_2S concentration higher than the level in the ambient medium is conceivable. The concentration gradient across the gill surface then would allow some H_2S to move by diffusion from the blood into the water, thus additionally reducing the toxicity of the dissolved sulfide. It is thus concluded that the acute toxicity to fathead minnows of free cyanide and dissolved sulfide solutions does not depend entirely on the concentration of ambient molecular HCN or H_2S, but that the CN^- and HS^- anions penetrate the gill epithelium less readily than do the molecular forms and contribute to the toxicities of these solutions increasingly as the pH increased.

The equations expressing the theory proposed by Tabata, with K as the basic ionization constant and $[H^+]$ replaced by $[OH^-]$, can be used to analyze the ammonia bioassay data of Lloyd and Herbert (1960). The calculated T_m/T_i ratios presented in Table 6 average 82 when the results for the tests at pH 7.0 with a very high CO_2 level are omitted. Therefore, the effective toxicity to the rainbow trout of the NH_3 molecule in solution is apparently about 82 times that of the NH_4^+ anion. The predicted LC50 values in Table 6 were estimated on the basis of the LC50 determined at pH 7.8 and a T_m/T_i ratio of 82. Only at pH 7.0 was there a marked difference between the

Table 6. Calculated T_m/T_i ratios and predicted LC50 values for ammonia bioassays with rainbow trout at different pH levels and assumed mean temperature of 19 C (data from Lloyd and Herbert (1960)

Test no.	pH	Free CO_2, mg/l	T_m/T_i ratio for test number [1]			Determined 500 min LC50,[2] mg/l as N		Predicted total ammonia LC50[3] mg/l as N
			1	2	3	NH_3	Total ammonia	
1	8.20	3.2	--	--	--	0.84	15.2	14.1
2	7.80	7.7	56	--	--	0.62	27.2	27.2
3	7.37	21.5	81	109	--	0.42	48.8	45.6
4	7.00	48.0	337	815	-1201	0.49	133	59.6

[1] Ratio applies to comparison of the test indicated by the test number below with the test at a higher pH whose test number appears in the first column. In the formula for computation of T_m/T_i, $[H^+]$ is replaced by $[OH^-]$ and $K_b = 1.695 \times 10^{-5}$ at 19 C.

[2] Assumed factor for proportion of total ammonia as molecular NH_3 of $1/(1 + 10^{pK_a - pH})$ with pK_a at 19 C equal to 9.432 (Robinson and Stokes 1955).

[3] LC50' calculations based on T_m/T_i of 82, test 2, and K_b, with $[H^+]$ replaced by $[OH^-]$ in the formula.

determined and predicted total ammonia LC50 values. At this low test pH associated with a high CO_2 tension, the blood pH was most likely lower than that of the fish tested at pH 7.8. The percentage of total ammonia present in the molecular form is thus decreased, and if NH_3 is the major internal toxic form, the observed LC50 at pH 7.0 should be greater than the predicted value. Therefore, the correct explanation of ammonia toxicity may be similar to that proposed for cyanide and sulfide, whose ionic forms are believed to penetrate the gill and to have a measurable toxicity considerably less than that of the respective molecular forms. However, because of involvement of ammonia in active exchange at the gill, a complete explanation may be more complicated, awaiting further physiological and toxicological investigation.

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APPENDIX A

DERIVATION OF EQUATIONS PROPOSED BY TABATA

Assuming a weak acid HA dissociates to give H^+ and A^-, then

$$\frac{[H^+][A^-]}{[HA]} = \text{ and at one pH, } \frac{[A^-]}{[HA]} = \frac{K_a}{[H^+]} \text{ and at pH', } \frac{[A^-]'}{[HA]'} = \frac{K_a}{[H^+]'} \qquad (1)$$

To account for the relationship between pH variations and weak acid toxicity, Tabata proposed the following formula whaere the toxicity of 1 mole of molecular form (T_m) and that of 1 mole of ionized form (T_i) is given by:

$$\frac{C}{LC50} = [HA] \cdot T_m + [A^-] \cdot T_i \qquad (2)$$

The left hand side of the equation (2) expresses the total toxicity of the weak acid while the first term on the right hand side stands for the toxicity due to the molecular form; the second term expresses the toxicity attributable to the ionic form. C is the proportionality constant.

The ratio of the toxicity of the molecular form to that of the ionized form (T_m/T_i) can be obtained from equations (1) and (2) by determining the LC50 at one pH, here referred to simple as pH, and the LC50' at another, here designated by the symbol pH'.

$$\begin{aligned} &\text{At pH} \quad C = [HA] + [A^-] \quad \text{and at} \\ &\text{pH'} \quad C' = [HA]' + [A^-]' \end{aligned} \qquad (3)$$

Therefore: $$\frac{[HA] + [A^-]}{LC50} = [HA] \cdot T_m + [A^-] \cdot T_i \quad \text{and} \tag{4}$$

$$\frac{[HA]' + [A^-]'}{LC50'} = [HA]' \cdot T_m + [A^-]' \cdot T_i$$

By equating the two expressions above, the relationship for T_m/T_i can be defined as follows:

$$\frac{HA + A^-}{LC50([HA] \cdot T_m + [A^-] \cdot T_i)} = \frac{[HA]' + [A^-]'}{LC50'([HA]' \cdot T_m + [A^-]' \cdot T_i)}$$

$$\frac{LC50'([HA]' \cdot T_m + [A^-]' \cdot T_i)}{LC50([HA] \cdot T_m + [A^-] \cdot T_i)} = \frac{[HA]' + [A^-]'}{[HA] + [A^-]}$$

$$([HA] + [A^-])\left[LC50'([HA]' \cdot T_m + [A^-]' \cdot T_i)\right] = ([HA]' + [A^-]')\left[LC50([HA] \cdot T_m + [A^-] \cdot T_i\right]$$

$$([HA] + [A^-])([HA]' \cdot LC50' \cdot T_m + [A^-]' \cdot LC50' \cdot T_i) = ([HA]' + [A^-]')([HA] \cdot LC50 \cdot T_m + [A^-] \cdot LC50 \cdot T_i)$$

$$([HA] + [A^-])([HA]' \cdot LC50' \cdot T_m) + ([HA] + [A^-])([A^-]' \cdot LC50' \cdot T_i) = ([HA]' + [A^-]')([HA] \cdot LC50 \cdot T_m) + ([HA]' + [A^-]')([A^-] \cdot LC50 \cdot T_i)$$

$$([HA] + [A^-])([A^-]' \cdot LC50' \cdot T_i) - ([HA]' + [A^-]')([A^-] \cdot LC50 \cdot T_i) = ([HA]' + [A^-]')([HA] \cdot LC50 \cdot T_m) - ([HA] + [A^-])([HA]' \cdot LC50' \cdot T_m)$$

$$([HA][A^-]' \cdot LC50' \cdot T_i) + ([A^-][A^-]' \cdot LC50' \cdot T_i) - ([HA]'[A^-] \cdot LC50 \cdot T_i) - ([A^-]'[A^-] \cdot LC50 \cdot T_i) = ([HA]'[HA] \cdot LC50 \cdot T_m) + ([A^-]'[HA] \cdot LC50 \cdot T_m) - ([HA][HA]' \cdot LC50' \cdot T_m) - ([A^-][HA]' \cdot LC50' \cdot T_m)$$

$$T_i\Big[([HA][A^-]'\cdot LC50') + ([A^-][A^-]'\cdot LC50') - ([HA]'[A^-]\cdot LC50) - ([A^-]'[A^-]\cdot LC50)\Big] = T_m\Big[([HA]'[HA]\cdot LC50) + ([A^-]'[HA]\cdot LC50) - ([HA][HA]'\cdot LC50') - ([A^-][HA]'\cdot LC50')\Big]$$

$$\frac{T_m}{T_i} = \frac{([HA][A^-]'\cdot LC50') + ([A^-][A^-]'\cdot LC50') - ([HA]'[A^-]\cdot LC50) - ([A^-]'[A^-]\cdot LC50)}{([HA]'[HA]\cdot LC50 + ([A^-]'[HA]\cdot LC50) - ([HA][HA]'\cdot LC50') - ([A^-][HA]'\cdot LC50')}$$

$$\frac{T_m}{T_i} = \frac{([A^-][A^-]')(LC50' - LC50) + [HA][A^-]'\cdot LC50' - [HA]'[A^-]\cdot LC50}{([HA]'[HA])(LC50 - LC50') + [A^-]'[HA]\cdot LC50 - [A^-][HA]'\cdot LC50'}$$

$$\frac{T_m}{T_i} = \frac{\frac{[A^-][A^-]'}{[HA]'[HA]}(LC50' - LC50) + \frac{[A^-]'}{[HA]'}\cdot LC50' - \frac{[A^-]}{[HA]}\cdot LC50}{LC50 - LC50' + \frac{[A^-]'}{[HA]'}\cdot LC50 - \frac{[A^-]}{[HA]}\cdot LC50'}$$

$$\frac{T_m}{T_i} = \frac{\frac{K_a}{[H^+]}\cdot\frac{K_a}{[H^+]'}(LC50' - LC50) + \frac{K_a}{[H^+]'}\cdot LC50' - \frac{K_a}{[H^+]}\cdot LC50}{LC50 - LC50' + \frac{K_a}{[H^+]'}\cdot LC50 - \frac{K_a}{[H^+]}\cdot LC50'}$$

$$\frac{T_m}{T_i} = \frac{\frac{K_a}{[H^+]'}\cdot LC50' + \frac{K_aK_a}{[H^+][H^+]'}\cdot LC50' - \frac{K_a}{[H^+]}\cdot LC50 - \frac{K_aK_a}{[H^+][H^+]'}\cdot LC50}{LC50 + \frac{K_a}{[H^+]'}\cdot LC50 - LC50' - \frac{K_a}{[H^+]}\cdot LC50'}$$

$$\boxed{\frac{T_m}{T_i} = \frac{\frac{K_a}{[H^+]'}(1 + \frac{K_a}{[H^+]})LC50' - \frac{K_a}{[H^+]}(1 + \frac{K_a}{[H^+]'})\cdot LC50}{(1 + \frac{K_a}{[H^+]'})\ LC50 - (1 + \frac{K_a}{[H^+]})LC50'} \qquad (5)}$$

The above equation (5) can be modified to estimate a new LC50' for a new pH' from the given pH, LC50, and T_m/T_i value.

$$\frac{K_a}{[H^+]'}(1 + \frac{K_a}{[H^+]})LC50'\cdot T_i - \frac{K_a}{[H^+]}(1 + \frac{K_a}{[H^+]'})LC50\cdot T_i =$$

$$(1 + \frac{K_a}{[H^+]'})LC50\cdot T_m - (1 + \frac{K_a}{[H^+]})LC50'\cdot T_m$$

$$\frac{K_a}{[H^+]'}\cdot LC50'\cdot T_i + \frac{K_aK_a}{[H^+]'[H^+]}\cdot LC50'T_i - \frac{K_a}{[H^+]}\cdot LC50\cdot T_i - - \frac{K_aK_a}{[H^+][H^+]'}\cdot LC50\cdot T_i =$$

$$LC50\cdot T_m + \frac{K_a}{[H^+]'}\cdot LC50\cdot T_m - LC50'\cdot T_m - \frac{K_a}{[H^+]}\cdot LC50'\cdot T_m$$

$$\frac{K_a}{[H^+]'}\cdot LC50'\cdot T_i + \frac{K_aK_a}{[H^+]'[H^+]}\cdot LC50'\cdot T_i + LC50'\cdot T_m + \frac{K_a}{[H^+]}\cdot LC50'\cdot T_m =$$

$$LC50\cdot T_m + \frac{K_a}{[H^+]'}\cdot LC50\cdot T_m + \frac{K_a}{[H^+]}\cdot LC50\cdot T_i + \frac{K_aK_a}{[H^+][H^+]'}\cdot LC50\cdot T_i$$

$$LC50' \left(\frac{K_a}{[H^+]'} \cdot T_i + \frac{K_a K_a}{[H^+]'[H^+]} \cdot T_i + T_m + \frac{K_a}{[H^+]} \cdot T_m \right) =$$

$$LC50 \left(T_m + \frac{K_a}{[H^+]'} \cdot T_m + \frac{K_a}{[H^+]} \cdot T_i + \frac{K_a K_a}{[H^+][H^+]'} \cdot T_i \right)$$

$$LC50' = \frac{\left(T_m + \frac{K_a}{[H^+]'} \cdot T_m + \frac{K_a}{[H^+]} \cdot T_i + \frac{K_a K_a}{[H^+][H^+]'} \cdot T_i \right)}{\left(\frac{K_a}{[H^+]'} \cdot T_i + \frac{K_a K_a}{[H^+]'[H^+]} \cdot T_i + T_m + \frac{K_a}{[H^+]} \cdot T_m \right)} \cdot LC50$$

$$LC50' = \frac{\left(\frac{T_m}{T_i} + \frac{K_a}{[H^+]'} \cdot \frac{T_m}{T_i} + \frac{K_a}{[H^+]} + \frac{K_a K_a}{[H^+][H^+]'} \right)}{\left(\frac{T_m}{T_i} + \frac{K_a}{[H^+]} \cdot \frac{T_m}{T_i} + \frac{K_a}{[H^+]'} + \frac{K_a K_a}{[H^+]'[H^+]} \right)} \cdot LC50$$

$$\boxed{LC50' = \frac{\left(1 + \frac{K_a}{[H^+]'}\right) \left(\frac{T_m}{T_i} + \frac{K_a}{[H^+]} \right)}{\left(1 + \frac{K_a}{[H^+]}\right) \left(\frac{T_m}{T_i} + \frac{K_a}{[H^+]'} \right)} \cdot LC50} \qquad (6)$$

THE ACUTE TOXICITY OF NITRITE TO FISHES

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INTRODUCTION

Only in the past few years has nitrite received much attention in toxicity studies by fisheries biologists, perhaps because it is generally present in only trace amounts in most natural freshwater systems. It is an intermediate product in the conversion of ammonia to nitrate by the nitrification process. In this process nitrosomonads convert ammonia to nitrite, and nitrobacters convert nitrite to nitrate. In a relatively stable situation, the first conversion, i.e. that of ammonia to nitrite, is the rate-limiting step in the total process. However, if something occurs to disrupt the stability of the process, such as a malfunction at a sewage treatment plant, or extremely low ambient temperatures, then nitrite may be discharged into, or produced in, the receiving water at a level which may be toxic to fishes. Water reuse systems using the nitrification process may also malfunction, resulting in increased nitrite levels in the treated water. Although these increased nitrite levels may be a short-lived phenomenon, nonetheless the highly toxic nature of nitrite to fishes warrants consideration.

Anthonisen and coworkers (1976) have demonstrated that a nitrite buildup can occur due to inhibition of the nitrification process by nitrous acid and un-ionized ammonia. Un-ionized ammonia inhibits nitrobacters at much lower concentrations than those at which it inhibits nitrosomonads (0.1 - 1.0 vs. 10 - 150 mg/l). Nitrous acid inhibits both nitrobacters and nitrosomonads at concentrations between 0.22 and 2.8 mg/l. When nitrite oxidation is inhibited, incomplete nitrification is observed, resulting in nitrite accumulation (Anthonisen et al. 1976).

Nitrite concentrations of 30 mg/l NO_2-N and higher have been reported by Klingler (1957) in receiving waters for effluents from metal, dye, and celluloid industries. McCoy (1972) has reported levels up to 73 mg/l NO_2-N in Wisconsin lakes and streams. In a reasonably clean cold water stream in Montana, we have occasionally found levels around 0.1 mg/l NO_2-N below a sewage treatment plant (Russo and Thurston 1974).

Until recently, only a small amount of information had been published on the toxicity of nitrite to fishes, and most of that literature dealt with static bioassays of 48 hours or less. However, in the last two years a greater number of papers have appeared. The available literature information is summarized in Table 1. There is a wide variation in the toxicity results reported. This range is probably attributable both to differences in water chemistry among the different investigators' experiments and to some genuine differences in susceptibility among fish species. McCoy (1972) tested 13 species, presumably using the same dilution water in all cases, and observed a wide variation in susceptibilities. There is also some indication that younger fish may be somewhat less susceptible to nitrite than are older fish of the same species (Russo et al. 1974, Smith and Williams 1974).

It is known that nitrite oxidizes hemoglobin (Hb) to methemoglobin (MetHb) (Bodansky 1951, Jaffé 1964, Kiese 1974). Thus, one way that nitrite is toxic to fishes is through formation of excessive amounts of MetHb which, unlike Hb, is incapable of transporting oxygen; MetHb in sufficiently high concentrations in fish blood can cause death. The percentage of total hemoglobin which is MetHb under normal conditions has been reported by Cameron (1971) to be 2.9% for wild rainbow trout (*Salmo gairdneri*) and 17% for hatchery-reared rainbow trout. Shterman (1970) has reported MetHb levels (as percent of total hemoglobin) in rainbow trout to be 2.7 - 3.9%; Brown and McLeay (1975) have reported levels of 0.9%, and Smith and Russo (1975) have reported levels of 3.6%. These values have been detected in the species when not under stress from environmental nitrite.

Smith and Williams (1974) observed elevated levels of MetHb in rainbow trout and chinook salmon (*Oncorhynchus tshawytscha*) exposed to nitrite. We have measured MetHb levels in 30-g rainbow trout exposed for 1-8 days to nitrite concentrations from 0.1 to 0.78 mg/l NO_2-N and found an increase in MetHb concentrations even at the lowest nitrite exposure: 14.3% MetHb (of total Hb) vs. 3.6% for controls (Smith and Russo 1975). Our results were comparable to those of Brown and McLeay (1975) who also found a significant rise in MetHb in rainbow trout exposed to 0.015 mg/l NO_2-N for four days; mortalities were observed at 0.2 and 0.3 mg/l, at which 80% of the fish blood Hb was in the MetHb form. Brown and McLeay also observed a decrease in total Hb at NO_2-N concentrations above 0.1 mg/l. In contrast to the above results, Cameron (1971) observed no change in MetHb content of rainbow trout blood after two days' exposure to 2 mg/l NO_2^- (0.61 mg/l NO_2-N).

This paper reports on some additional acute toxicity studies we have conducted with rainbow trout, including an investigation of the effect of chloride ion on nitrite toxicity. Some data on the toxicity of nitrite to fathead minnows (*Pimephales promelas*) and mottled sculpins (*Cottus bairdi*) are also presented.

MATERIALS AND METHODS

The bioassays were conducted either in plastic tanks containing 64 liters of water with a replacement time of 5-6 hours and using proportional diluters (Mount and Brungs 1967) for toxicant delivery, or in fiberglass tanks containing 350 liters of water with a replacement time of 1.3 hours and

TABLE 1. SUMMARY OF LITERATURE DATA ON NITRITE TOXICITY TO FISHES.

Reference	Fish and Size	Type of Bioassay	Temp. (°C)	pH	Type of Water (mg/liter)	NO_2-N (mg/liter)	Results Reported
Gillette et al. (1952)	creek chub (*Semotilus a. atromaculatus*), 3-4 inches	static	15-21	8.3	Hardness 98.0	80-400[a]	critical range
Wallen et al. (1957)	mosquitofish (*Gambusia affinis*), adult female	static	21-24	7.1-7.5	Alkalinity <100 Turbidity 120-140	1.6[a]	24-hr LC50
		"	"	"	"	1.5[a]	48- and 96-hr LC50
Klingler (1957)	minnow (*Phoxinus laevis*), 5-8 cm	partial static	18-21	NR[b]	NR	10[a]	fatal in 14 days
		"	"	NR	NR	2030[a]	fatal in 1.5 hr
McCoy (1972)[c]	logperch (*Percina caprodes*)	static	NR	NR	NR	5	mortality in <3 hr
	brook stickleback (*Culaea inconstans*)	"	NR	NR	NR	5	mortality in 3-5 hr
	carp (*Cyprinus carpio*)	"	NR	NR	NR	40	no mortality in 48 hr
		"	NR	NR	NR	100	mortality in 45 hr
	black bullhead (*Ictalurus melas*)	"	NR	NR	NR	40	no mortality in 48 hr
		"	NR	NR	NR	100	mortality in 24 hr
	common white sucker (*Catostomus commersoni*)	"	NR	NR	NR	100	survived for 48 hr
	quillback (*Carpiodes cyprinus*)	"	NR	NR	NR	100	survived for 36 hr
Smith and Williams (1974)	rainbow trout (*Salmo gairdneri*) 100 g	flow-through	10	7.9	Hardness 200	0.55	55% mortality in 24 hr
	4.5 g	"	"	"	"	1.60	50% mortality in 24 hr
	chinook salmon (*Oncorhynchus tshawytscha*) 32 g	"	"	"	"	0.50	40% mortality in 24 hr
Westin (1974)	chinook salmon (*Oncorhynchus tshawytscha*) 1.50-10.55 g	partial static	13.6-15.6	6.8-7.2[d]	NR	0.88[a]	96-hr LC50
		"	"	"	NR	0.73[a]	7-day LC50

(continued)

TABLE 1. Continued.

Reference	Fish and Size	Type of Bioassay	Temp. (°C)	pH	Type of Water (mg/liter)	NO_2-N (mg/liter)	Results Reported
Colt (1974), Colt and Tchobanoglous (1976)	channel catfish (*Ictalurus punctatus*), fingerlings	static	30	8.6-8.8	Alkalinity 220	13[a]	96-hr LC50
Russo, et al. (1974)	rainbow trout (*Salmo gairdneri*) 2 g	flow-through	10.8	7.9	Alkalinity 176 Hardness 199	0.39	96-hr LC50
	12-14 g	"	11.6-12.6	"	"	0.19-0.27	96-hr LC50
	235 g	"	9.5	"	"	0.20	96-hr LC50
	12 g	"	12.4	"	"	0.14-0.15	Asymptotic LC50 (8-19 days)
Brown and McLeay (1975)	rainbow trout (*Salmo gairdneri*) 9 g	flow-through	12	6.4-6.7	Alkalinity 2-8 Hardness 3-9	0.23	96-hr LC50
Konikoff (1973, 1975)	channel catfish (*Ictalurus punctatus*) 40 g	static	21	7.4-7.8	Alkalinity 60-70	7.5[a]	96-hr TL_m
Thurston, et al. (To be submitted)	cutthroat trout (*Salmo clarki*) 1 g	flow-through	12.4	7.85,7.88	Alkalinity 178 Hardness 200	0.38,0.37	36-day LC50
	3 g	"	11.8,12.1	7.88,7.80	"	0.56,0.48	96-hr LC50
	1-3 g	"	11.8-12.4	7.80-7.88	"	0.4	Asymptotic LC50

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[a] Calculated from $NaNO_2$ or NO_2^- data reported by author.

[b] NR = not reported by author.

[c] The following species were also tested, and survived less than 12-24 hr in 20-40 mg/liter NO_2-N: Johnny darter (*Etheostoma nitrum*), bluegill (*Lepomis macrochirus*), pumpkinseed (*Lepomis gibbosus*), spotfin shiner (*Notropis spilopterus*), sand shiner (*Notropis stramineus*), hog sucker (*Hypentelium nigricans*), stonecat (*Noturus flavus*); all fishes tested were small fingerlings or minnows.

[d] D. T. Westin, personal communication.

using metering pumps for toxicant delivery. Reagent grade $NaNO_2$ and NaCl were used. Five test tanks plus a control tank were used in all cases, with either 10 or 20 fish in each tank. The rainbow trout used were hatchery-reared fish obtained from the Bozeman (Montana) Fish Cultural Development Center, U. S. Fish and Wildlife Service. They were reared to test size in water from the same ground spring source as that which was subsequently used as the test dilution water. The fathead minnows were obtained from the Miles City (Montana) Hatchery, U. S. Fish and Wildlife Service. The sculpins were collected from Rocky Creek (Gallatin County), Montana. Fish were acclimated to the test tanks for at least two days prior to toxicant introduction and were not fed during acclimation or testing. Fish which died during the test were individually weighed and measured within 0-8 hours. Survivors were measured at the termination of the test.

Nitrite concentrations were determined by the method described by EPA (1974). Dissolved oxygen was measured either using the azide modification of the iodometric method (American Public Health Association 1976) with phenylarsine oxide substituted for sodium thiosulfate, or with a Yellow Springs Instrument Co. Model 54-RC meter. Temperature was measured with a certified thermometer, and pH with a Beckman Phasar-I meter. All other chemical analyses were performed according to the procedures of the American Public Health Association (1976). All colorimetric measurements were made on a Varian 635 ultraviolet-visible spectrophotometer.

Averages and ranges of values for the tank water over all tests were: dissolved oxygen 8.9 (7.9 - 10.0) mg/l; alkalinity 177 (171 - 191) mg/l $CaCO_3$; hardness 199 (188 - 207) mg/l $CaCO_3$; NH_3-N 0.00 (0.00 - 0.07) mg/l; NO_3-N 0.08 - 6.85 mg/l; Cl^- 0.35 (0.00 - 0.74) mg/l. The range of NO_2-N concentrations for all tests, and the Cl^- concentrations for those tests in which Cl^- was a test variable, are reported in Tables 2 and 3. Temperature and pH values for each test are also given in Tables 2 and 3.

Median lethal concentration (LC50) values and their 95% confidence limits were calculated from the experimental data using the trimmed Spearman-Karber method (Hamilton et al. 1977).

RESULTS AND DISCUSSION

The results of five 96-hr bioassays on rainbow trout are presented in Table 2 and Figure 1. The 53- and 60-g fish were from the same lot; the 21-, 24- and 188-g fish were from a second lot. Although the two lots of fish were tested approximately 10 months apart, there is good agreement among all five tests, with 96-hr LC50 values ranging from 0.19 to 0.28 mg/l NO_2-N. In an earlier report on nitrite toxicity (Russo et al. 1974), four other 96-hr bioassays on rainbow trout (size range 12 - 235 g) gave LC50 values of 0.19 - 0.27 mg/l NO_2-N. Although the fish sizes in that earlier paper covered a wider range than those reported here, the LC50 values were within the range reported in the present study. The average 96-hr LC50 for all nine tests on rainbow trout within the size range 12 - 235 g is 0.24 mg/l NO_2-N (range 0.19 - 0.28). The highest concentrations tested where no mortalities occurred ranged between 0.06 and 0.13 mg/l NO_2-N. However, earlier work (Russo et al. 1974) on 2-g and sac fry rainbow trout showed that these smaller fish were

TABLE 2. RESULTS OF ACUTE NITRITE BIOASSAYS ON RAINBOW TROUT (*SALMO GAIRDNERI*), FATHEAD MINNOW (*PIMEPHALES PROMELAS*), AND MOTTLED SCULPIN (*COTTUS BAIRDI*).

Test No.	Avg Fish Size		Concentration Range Tested (mg/ℓ NO_2-N)	LC50 (95% C.I.) (mg/ℓ NO_2-N)		Temp (C) Avg (Range)	pH Avg (Range)
	Wt (g)	Length (cm)		72 hr	96 hr		
				RAINBOW TROUT			
323	20.6	11.8	0.22-1.70	0.29 (0.24-0.36)	--	10.1 (10.0-10.2)	8.05 (7.94-8.12)
326	24.3	12.3	0.08-0.59	0.32 (0.27-0.38)	0.28 (0.24-0.33)	10.2 (10.1-10.3)	8.10 (7.99-8.33)
243	53.1	15.7	0.08-0.48	0.35 (0.29-0.41)	0.27 (0.22-0.33)	9.8 (9.7-10.0)	7.68 (7.58-7.79)
244	60.5	16.6	0.11-0.78	0.30 (0.25-0.36)	0.27 (0.23-0.32)	9.8 (9.7-9.9)	7.76 (7.71-7.83)
423	188	23.6	0.10-0.79	0.22 (0.17-0.28)	0.19 (0.15-0.25)	10.4 (10.3-10.7)	7.81 (7.76-7.86)
				FATHEAD MINNOW			
346	2.3	6.2	2.26-7.29	5.54 (3.66-7.95)	2.99 (2.35-3.81)	13.0 (12.7-13.2)	8.05 (8.03-8.09)
349	2.3	6.4	2.30-7.52	3.94 (2.37-6.55)	2.30	12.7 (12.5-12.8)	8.04 (7.96-8.10)
				MOTTLED SCULPIN			
315	1.8	5.4	0.82-2.66	No mortalities in 96 hr		12.8 (12.3-13.9)	8.10 (7.93-8.19)
316	2.0	--	2.68-8.75	No mortalities in 96 hr		13.1 (12.6-13.6)	8.06 (8.03-8.13)
319	2.3	--	8.41-26.3	No mortalities in 72 hr		13.2 (12.6-14.3)	8.14 (8.09-8.19)
348	1.6	5.2	21.6-66.7	No mortalities in 154 hr		13.6 (13.1-14.1)	8.08 (8.00-8.20)

TABLE 3. RESULTS OF ACUTE NITRITE BIOASSAYS ON RAINBOW TROUT (*SALMO GAIRDNERI*) WITH ADDITION OF CHLORIDE ION.

Test No.	Avg Fish Size Wt (g)	Avg Fish Size Length (cm)	Concentration Range Tested (mg/ℓ NO_2-N)	Cl^- Concn (mg/ℓ)	96-hr LC50 (95% C.I.) (mg/ℓ NO_2-N)	Temp (C) Avg (Range)	pH Avg (Range)
357	69.5	16.7	0.16-1.08	1.2	0.46 (0.36-0.58)	10.4 (10.3-10.5)	7.92 (7.87-7.99)
362	69.1	17.0	0.88-5.99	5.1	2.36 (1.86-3.00)	10.4 (10.4-10.5)	8.01 (7.96-8.05)
363	79.0	17.6	0.97-6.74	10.4	3.54 (2.90-4.32)	10.4 (10.2-10.6)	7.90 (7.83-7.96)
366	86.4	18.4	1.56-10.76	20.2	6.69 (5.64-7.93)	10.5 (10.4-10.5)	7.84 (7.80-7.88)
369	99.3	19.4	2.57-18.33	40.9	12.2 (8.96-18.4)	10.3 (10.0-10.5)	7.74 (7.71-7.78)
377	113	20.0	4.92-33.90	40.8	12.5 (9.96-16.0)	10.4 (10.2-10.5)	7.69 (7.67-7.72)

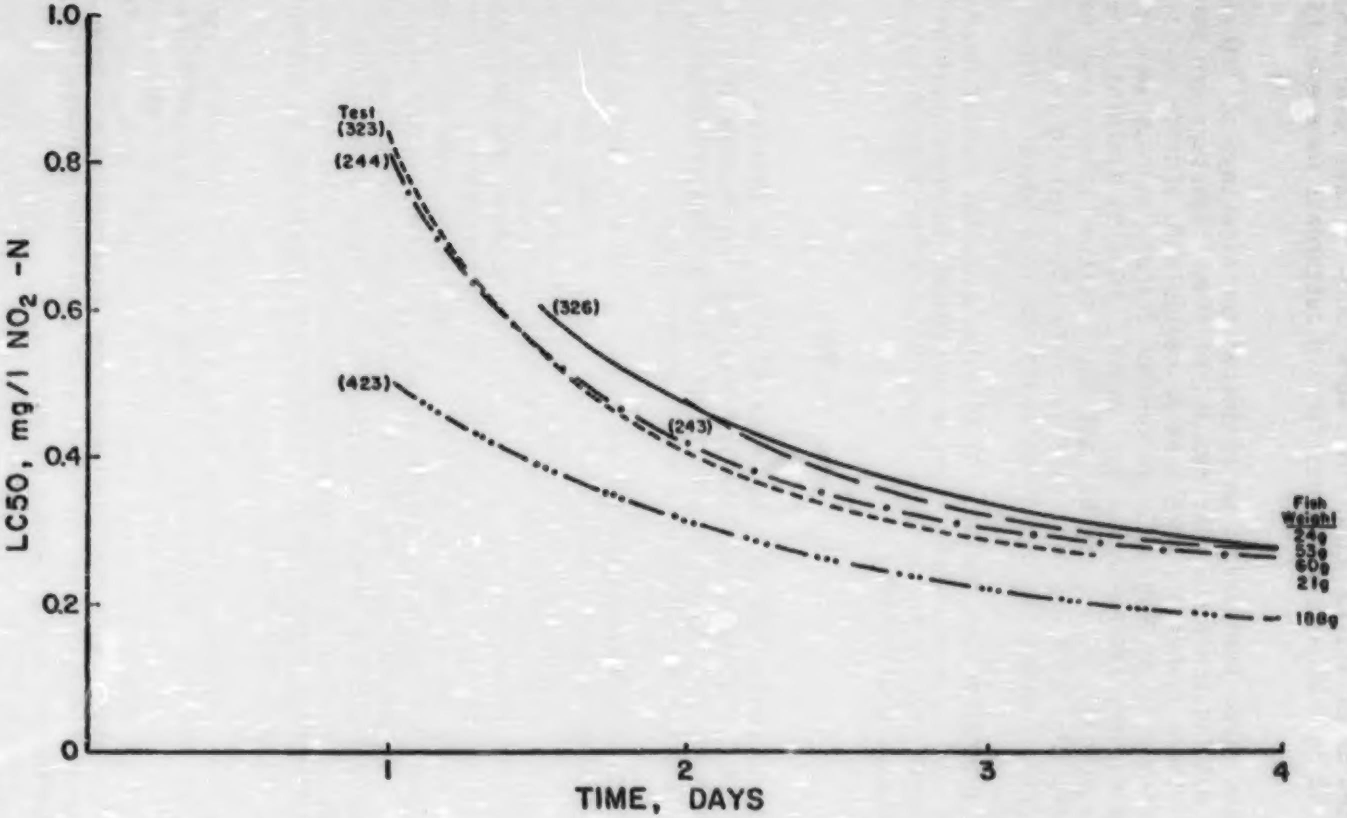

Figure 1. Acute toxicity of nitrite to rainbow trout (*Salmo gairdneri*) (pH 7.7-8.1, temp. 9.8-10.4°C, Cl^- ≤ 0.4 mg/ℓ).

somewhat less susceptible to nitrite. Also, 3-g cutthroat trout (*Salmo clarki*) tested under similar conditions were found to have 96-hr LC50 values averaging 0.52 mg/l NO_2-N, and values for 1-g cutthroats were even higher (Table 1).

On fishes other than trout, we conducted two bioassays on fathead minnows and four on mottled sculpins. The results of these bioassays are summarized in Table 2. These fishes are much less susceptible to nitrite toxicity than the rainbow trout. The 96-hr LC50 values for fathead minnows were an order of magnitude higher, averaging 2.6 mg/l NO_2-N. Mottled sculpins were tested successively using 3, 9, 26, and 67 mg/l NO_2-N as the highest test concentrations. In all four of these bioassays no mortalities were observed throughout the test periods (except for one anomalous death in 4 hours at 6 mg/l).

Having determined the toxicity of nitrite to rainbow trout under a given set of water quality conditions, we were interested in investigating the effects of variations in those conditions. Nitrite ion establishes the following aqueous equilibrium:

$$NO_2^- + H^+ \rightleftarrows HNO_2$$

This equation suggests that pH might have an effect on nitrite toxicity if either of these two chemical species (HNO_2 or NO_2^-) were more or less toxic than the other, or if they acted synergistically or antagonistically. A pH decrease would cause an increase in HNO_2 concentration.

For a total NO_2-N concentration of 1 mg/l at pH 8.5, and using a pK_a of 3.29, the NO_2^- concentration is 7.14×10^{-5} M and the HNO_2 concentration is 4.37×10^{-10} M. For the same total NO_2-N concentration at pH 7.5, the NO_2^- concentration is 7.14×10^{-5} M and the HNO_2 concentration is 4.37×10^{-9} M. For both of these cases, and for the pH range in between, the NO_2^- concentration remains essentially constant, whereas the HNO_2 concentration varies by an order of magnitude. Even so, the NO_2^- concentration remains 4-5 orders of magnitude higher than the HNO_2 concentration, and because total nitrite is toxic at such relatively low levels, it is difficult to conceive that the lesser of the two nitrite chemical species (i.e., HNO_2) is the principal toxic form. However, inasmuch as the concentration of HNO_2 does vary by an order of magnitude within the pH range in question, it is reasonable to assume that its toxic effect might be measurable.

To test this hypothesis, we conducted a series of nitrite bioassays in which we varied the pH. Details of this study will be reported elsewhere, but the major conclusions reached were that nitrite toxicity to rainbow trout is independent of pH within the range tested (pH 7.5 - 8.5), and that nitrite toxicity is correlated with NO_2^- concentration (essentially the same as total nitrite concentration), and is not correlated with HNO_2 concentration. Thus, NO_2^- is the principal toxic species of nitrite.

During our pH variation experiments we observed that when hydrochloric acid (HCl) was used to lower the solution pH, the toxicity of nitrite was greatly decreased. This indicated that chloride ion (Cl^-) might be exerting an antagonistic effect on nitrite toxicity. We therefore conducted a series of nitrite bioassays where we added Cl^- (as NaCl) so that the Cl^- concentration in the test tanks was 1, 5, 10, 20 and 41 mg/l. The rainbow trout used in these bioassays were all from the same lot. The bioassay using 41 mg/l Cl^- was run in duplicate, and the duration of acclimation of the fish to the NaCl solutions, before addition of $NaNO_2$, was 5 days in one case and 10 in the other. Results of the two tests were in extremely close agreement, indicating that the difference in acclimation times between 5 and 10 days had little or no measurable effect. The results of these experiments are summarized in Table 3; the toxicity curves are presented in Figure 2 and include a zero-chloride bioassay for purposes of comparison.

It can be seen from the table and figure that Cl^- exerts a marked effect on nitrite toxicity; an increase in Cl^- concentration causes a decrease in nitrite toxicity. This effect is linearly correlated. Using a weighted regression analysis (where the observation is weighted as 1/variance of the LC50), we obtain the following correlation coefficients: for 48 hr, .9964 (p=.00000); for 72 hr, .9957 (p=.00000); for 96 hr, .9873 (p=.00003).

Comparison of LC50 values for two bioassays in which chloride ion concentration of 10 mg/l was achieved, in one case by addition of NaCl (Table 3, Test 363), and in the other case by addition of HCl (data not given here), indicates that Cl^- is exhibiting this inhibitory effect; there is no evidence that it is attributable to Na^+. Thus, we have established that Cl^- ion exhibits an antagonistic effect on nitrite toxicity. From preliminary results of other research in our laboratory, we have found that bromide ion exhibits a similar inhibitory effect. Other ions may also exhibit this kind of antagonism to nitrite toxicity.

CONCLUSIONS

The results of our nitrite bioassays on fishes (including pH variation data not yet published), and the reported results of others, lead us to conclude that: (a) Exposure to nitrite causes an increase in methemoglobin concentration in fish blood, although this may not be the only toxic action of nitrite on fishes. (b) There are differences in susceptibility to nitrite among fish species, with rainbow and cutthroat trouts being much more susceptible to nitrite than fathead minnows or sculpins. (c) Rainbow trout fry are less susceptible to nitrite than are larger rainbow trout; there is no readily apparent size-related difference in toxicity among rainbow trout between 12 and 235 g. (d) the toxicity of nitrite is related to nitrite ion concentration, not nitrous acid concentration; changes in pH in the range 7.5 - 8.5 do not affect nitrite toxicity. (e) An increase in chloride concentration causes a decrease in nitrite toxicity; this relationship is linear at least up to 40 mg/l Cl^-.

ACKNOWLEDGMENT

This work was funded by the U. S. Environmental Protection Agency, Duluth, Minnesota, Research Grants No. R800861 and R803950. Robert J. Luedtke and Charles Chakoumakos provided valuable assistance with the

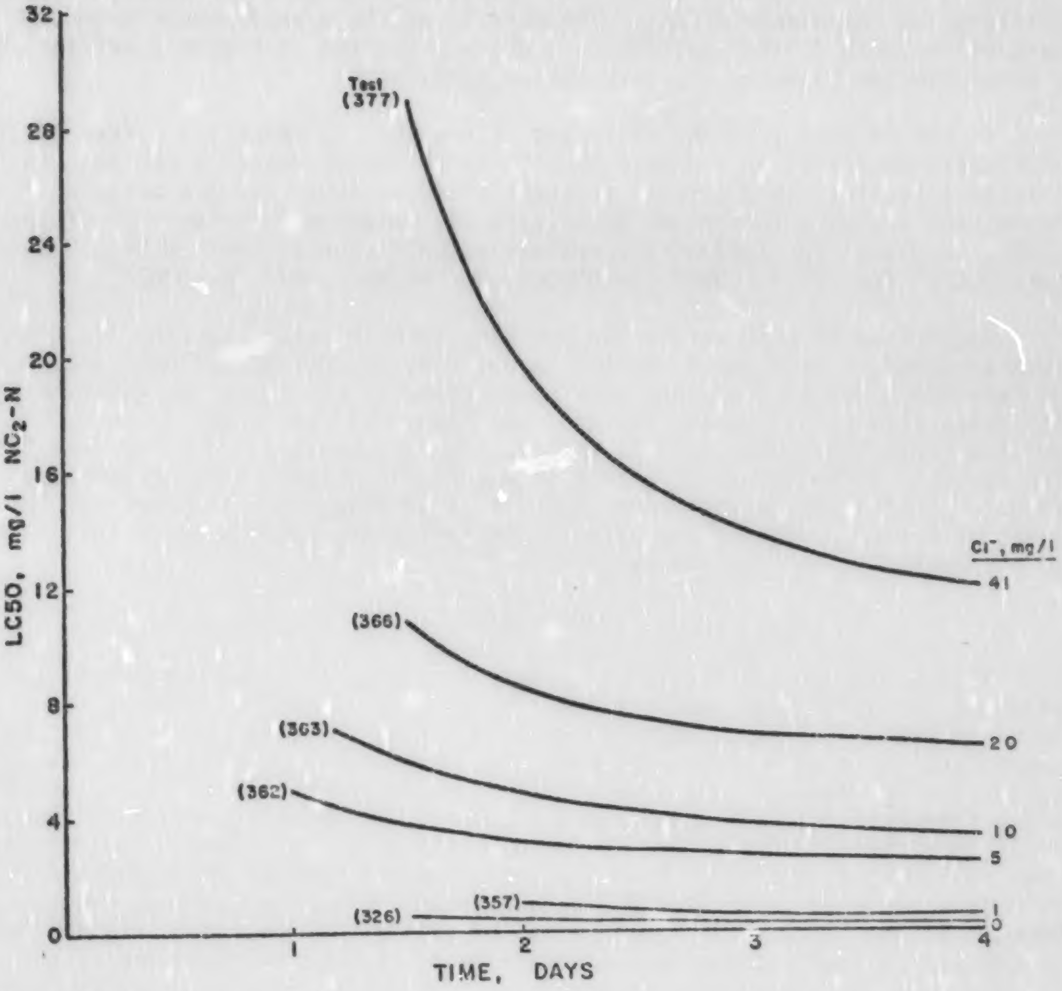

Figure 2. Effect of chloride on nitrite toxicity to rainbow trout (*Salmo gairdneri*).

biological procedures and chemical analyses. We thank Kenneth Emerson and Martin A. Hamilton for assistance with some of the chemical and statistical calculations.

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COPPER TOXICITY: A QUESTION OF FORM

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An abundance of literature indicates that copper toxicity is one of the more intensively investigated areas of fish toxicology. Much of the data on copper toxicity comes from acute toxicity studies on a wide variety of fish species, in waters of differing quality, using diverse methods. Compilations of these data can provide valuable information, but are little help in understanding and predicting toxic levels of copper. A second large area of research into copper toxicity involves studies whose goal is to increase the understanding of the role of receiving water quality on copper toxicity and from this understanding to generate a better predictive capability for estimating potentially adverse levels of copper in various natural waters. Although there are many factors which complicate this predictive capability (e.g. variable copper exposure levels, biological acclimatization, complex wastes, sublethal effects), it has generally been sought through relatively simple experiments relying on continuous short-term exposure at constant copper concentrations and utilizing death as the indicator.

Our interest in copper form was stimulated because of the toxicity of relatively low levels of copper in a series of flow through toxicity tests conducted at the Western Fish Toxicology Station (WFTS). The 96-hr LC50 values obtained in these tests with juvenile salmonids ranged from 15 to 38 μg/ℓ; these values were significantly lower than most related data in the literature. In my presentation today I wish briefly to trace the contemporary history of research into the effects of receiving water quality on copper toxicity and to present some recent data dealing with this subject. For additional information on the toxicity of copper to fish I recommend the critical review of the literature by Doudoroff and Katz (1953), the toxicity compendium of McKee and Wolf (1963), and the discussion and recommendations in Water Quality Criteria 1972 (Nat. Acad. Sci. and Nat. Acad. Engr. 1973).

In the latter report the primary effect noted of receiving water quality on copper toxicity was the effect of hardness. This effect is generally recognized, the best known reference being that of Lloyd and Herbert (1962). Their data (Figure 1) indicated that higher copper concentrations were required to produce lethality as the total hardness increased. When hardness

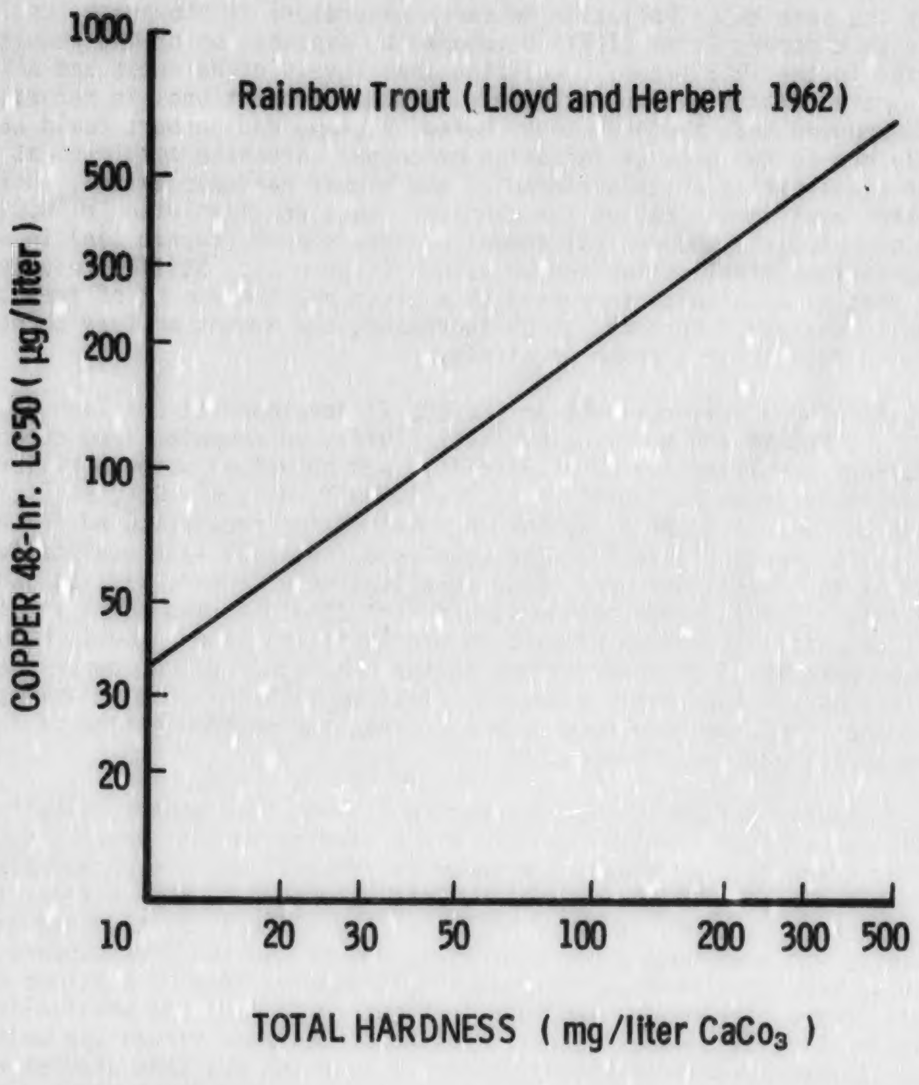

Figure 1. The relationship between total hardness and the 48-hr LC50 of copper to rainbow trout.

increased. When hardness increased over a range from 15 to 320 mg/ℓ as $CaCO_3$ the 48-hr LC50 for rainbow trout (*Salmo gairdneri*) increased from about 45 μg/ℓ to about 450 μg/ℓ.

Nine years after Lloyd and Herbert's 1962 paper, a related paper came out of the same Water Pollution Research Laboratory at Stevenage, England, and in this paper, Stiff (1971) developed an explanation of the results reported in the 1962 paper. Realizing that levels of hardness and alkalinity usually are related and approximately directly proportional in natural waters, Stiff proposed that the phenomenon noted by Lloyd and Herbert could have been largely due to the greater formation of copper carbonate complexes at the higher alkalinities which accompanied the higher hardness values. Utilizing published equilibrium values for chemical reactions involving Cu^{++} HCO_3^-, and H^+ he computed the theoretical amount of free copper (cupric ion) in waters having various alkalinities and pH values (Figure 2). Stiff's results indicated that as alkalinity increased at a given pH, the amount of free copper decreased sharply. Further, as pH increased, the amount of free copper also decreased greatly at a given alkalinity.

Utilizing a computer program (REDEQL 2) developed at Cal Tech by Morgan, Morel, and McDuff and modified by Ingel (1976), we computed free copper concentrations for a variety of alkalinities and pH values using equilibria data for reactions among Cu^{++} (10^{-6} M/ℓ), Ca^{++}, MG^{++}, NA^+, K^+, $CO_3^=$, SO_4^{++}, Cl^-, H^+, and OH^- when present in ratios recommended for reconstituted freshwaters for toxicity tests (Table 1). The results we obtained were qualitatively identical to Stiff's and very close quantitatively (Figure 2). It should be noted that Stiff ignored what he termed "the slowly formed complex" of copper in his computations and we allowed no precipitation in our model; both constraints were based on observations in the laboratory using copper specific ion electrodes. Apparently attaining final equilibrium concentrations of some copper complexes may require longer than the residence time of aquaria, mixing zones, and some rivers.

The matrix defined by the interactions among free copper, alkalinity, and pH can be simplified to a relationship similar to that described by the line for reconstituted freshwater shown in Figure 3. Since pH and alkalinity are rather closely related in most natural waters, i.e. high alkalinity and high pH occur together, it is possible to generalize a relationship between alkalinity and free copper (or conversely between pH and free copper). The alkalinity-pH relationships observed in 110 samples from 52 stations on 37 western Oregon streams are included in Figure 3 showing the pH-alkalinity regression line as well as lines enclosing the extreme values (Samuelson 1976). I draw these comparisons primarily to point out that studies which I will discuss in which we used these four reconstituted freshwaters are generally applicable to natural waters and do not refer solely to four arbitrary points in the pH-alkalinity matrix.

The reconstituted freshwaters to which I refer were recommended in "Methods for Acute Toxicity Tests with Fish, Macro-invertebrates, and Amphibians" (U. S. Environmental Protection Agency 1975). We decided to use these waters because we wanted maximum uniformity in water quality and we

TABLE 1. QUANTITIES OF REAGENT-GRADE CHEMICALS USED TO PREPARE RECOMMENDED RECONSTITUTED FRESH WATERS AND THE RESULTING WATER QUALITIES.[a]

Name	Salts Required (mg/ℓ)				Nominal Range and Observed Mean Value[b]		
	$NaHCO_3$	$CaSO_4 \cdot 2H_2O$	$MgSO_4$	KCl	pH	Hardness	Alkalinity
Very soft	12	7.5	7.5	0.5	6.4-6.8 (7.2)	10-13 (13)	10-13 (12)
Soft	48	30.0	30.0	2.0	7.2-7.6 (7.6)	40-48 (46)	30-35 (35)
Hard	192	120.0	120.0	8.0	7.6-8.0 (8.1)	160-180 (182)	110-120 (125)
Very Hard	384	240.0	240.0	16.0	8.0-8.4 (8.5)	280-320 (359)	225-245 (243)

[a] The Committee on Methods for Toxicity Tests with Aquatic Organisms, 1975.

[b] Mean value (in parenthesis) from bioassays.

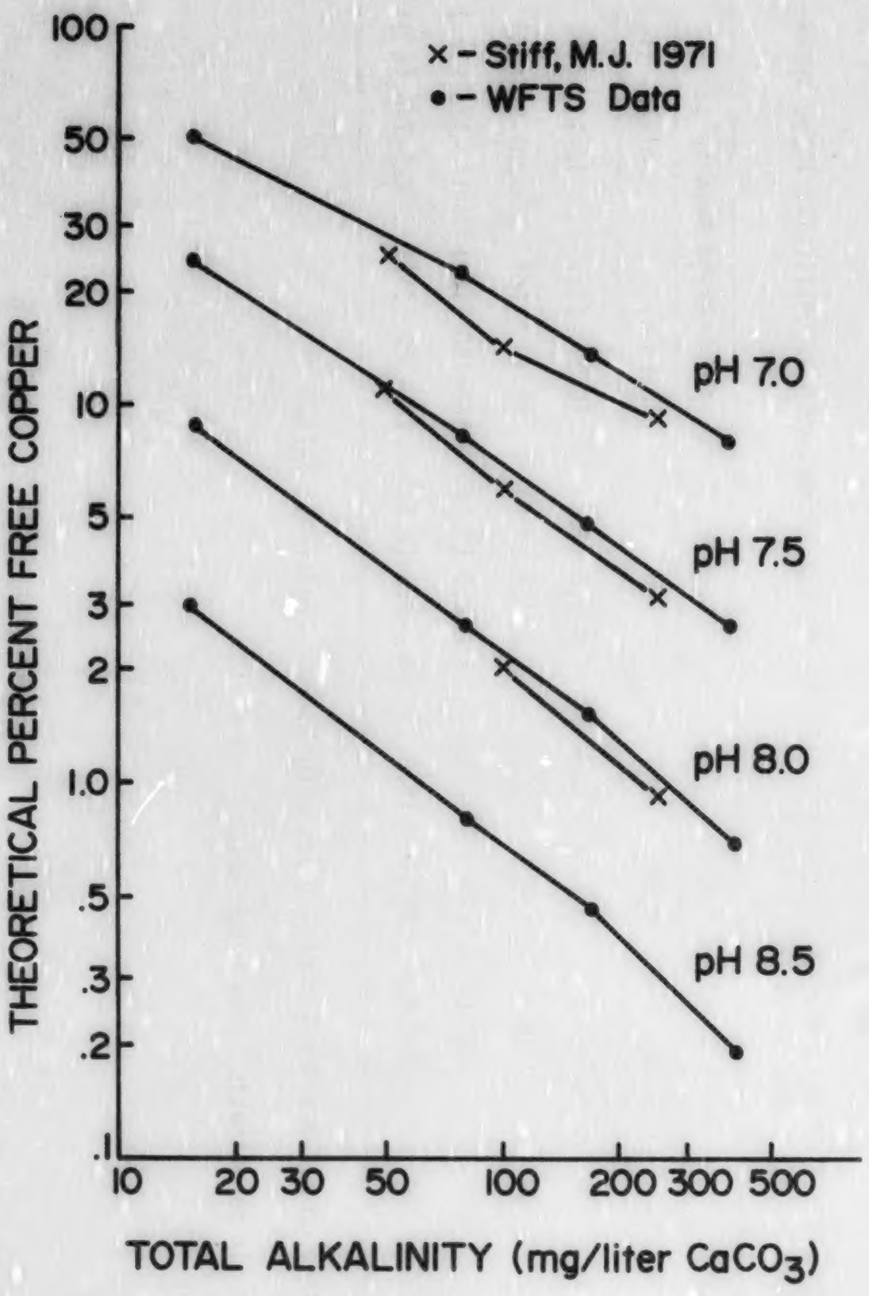

Figure 2. Calculated percent free copper (cupric ion) at indicated alkalinities and pH values.

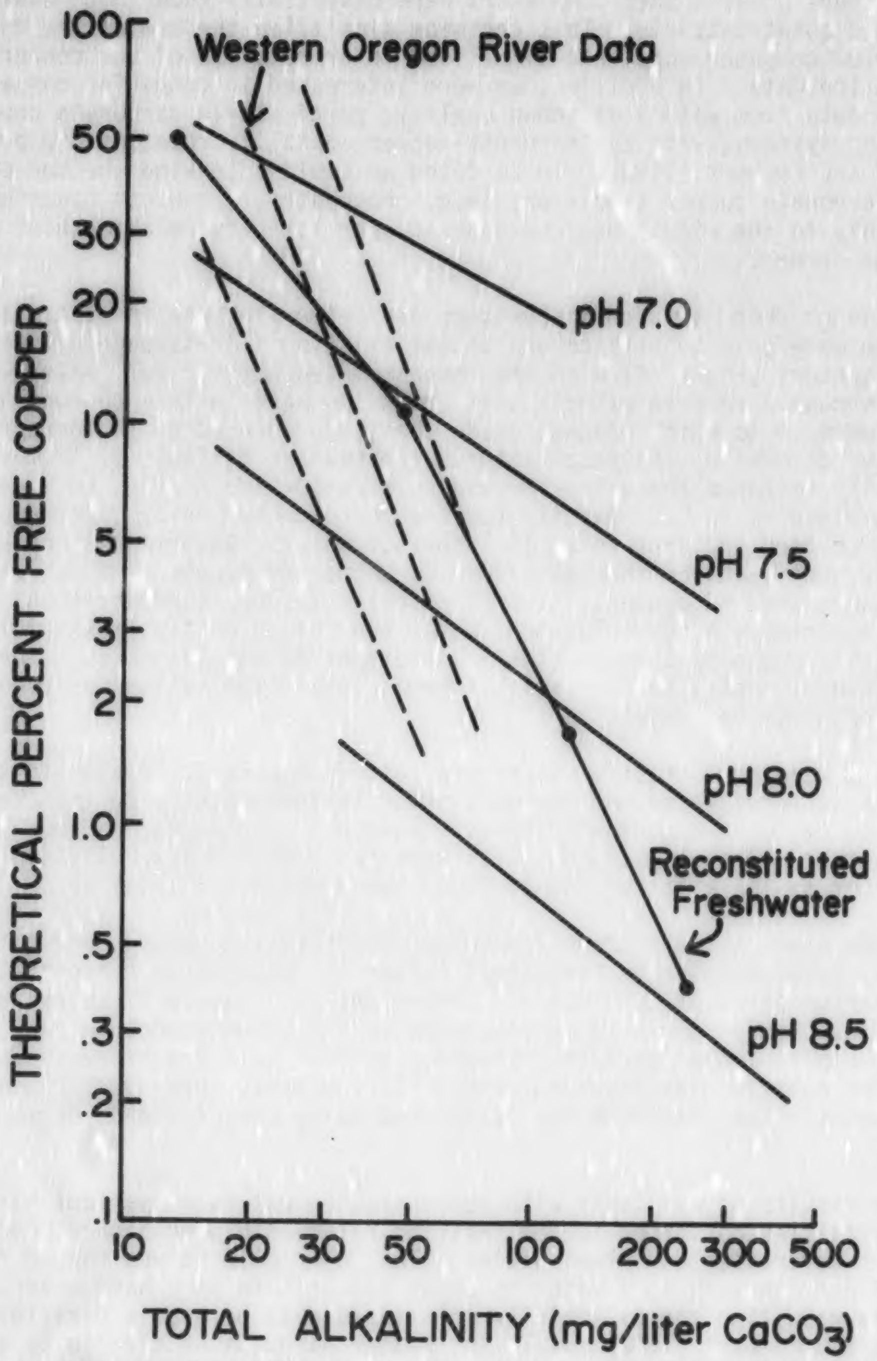

Figure 3. Relationship between pH, alkalinity, and theoretical percent free copper for natural waters and reconstituted freshwaters.

wanted waters whose copper complexors were essentially known both qualitatively and quantitatively. This decision simplified the use of the chemical equilibrium computer model and simplified interpretation of the copper specific ion data. In addition, we were interested in comparing copper toxicity data from waters of known quality, particularly carbonate copper complexing systems, with the hardness-copper mortality relationship published by Lloyd and Herbert (1962). In so doing we could determine whether effects of non-carbonate copper complexors (e.g. phosphates, organics) contributed appreciably to the widely used hardness-copper toxicity relationships of Lloyd and Herbert.

Although reconstituted freshwaters are primarily used in static toxicity tests, we were able to utilize one of our existing flow-through diluter diluter systems (Figure 4) with the reconstituted water. Well water was passed through a reverse osmosis unit producing water with a conductivity of about 1 umho/cm to which reagent grade chemicals were added in appropriate quantities to make up the reconstituted freshwaters in Table 1. Pumps continually agitated the water, providing aeration and mixing, and temperature was maintained at 12 C. Toxicity tests were conducted in 19 liter aquaria 92x26x41 cm deep and containing 14 liters of water. Aquaria were dosed with a diluter modified from that described by Mount and Brungs (1967). Twelve aquaria were used (6 concentrations X 2 replicates per concentration). Time for 50% aquarium volume replacement was 1 hour based on the flow-volume relationship shown by Sprague (1969). Photoperiod was set to coincide with sunrise-sunset tables for Corvallis, Oregon (dim illumination was used in lieu of complete darkness).

Tests were conducted for 96-hr and test fish were acclimated to the reconstituted freshwater for one week prior to the toxicity tests. Tests were conducted with 3-month-old chinook salmon (*Oncorhynchus tshawytscha*) having a mean weight of 1.35 g. Fish were fed Oregon Moist Pellet up to 48 hours prior to the start of the test, and were not fed thereafter.

Water analyses were conducted daily for dissolved oxygen, pH, total hardness, total alkalinity, and total copper at each copper concentration (one aquarium per duplicate pair). Copper analysis was by flameless atomic absorption. Daily cupric ion activity measurements were made *in situ* using an Orion cupric ion electrode*. In order to eliminate interference due to light, the aquarium was provided with a black plastic cover during cupric ion measurements. The electrode was calibrated using copper standards in acetate buffer.

The results of our tests with reconstituted waters of various hardnesses and alkalinities conformed to the familiar relationship of higher LC50 values at higher hardnesses and alkalinities. The 96-hr LC50 values ranged from about 10 μg/ℓ in very soft water to about 125 μg/ℓ in very hard water. We found the resulting copper toxicity relationship to be nearly parallel to that of Lloyd and Herbert (1962), utilizing either hardness or alkalinity as the determinant (Figure 5). This result indicated that the hardness-copper

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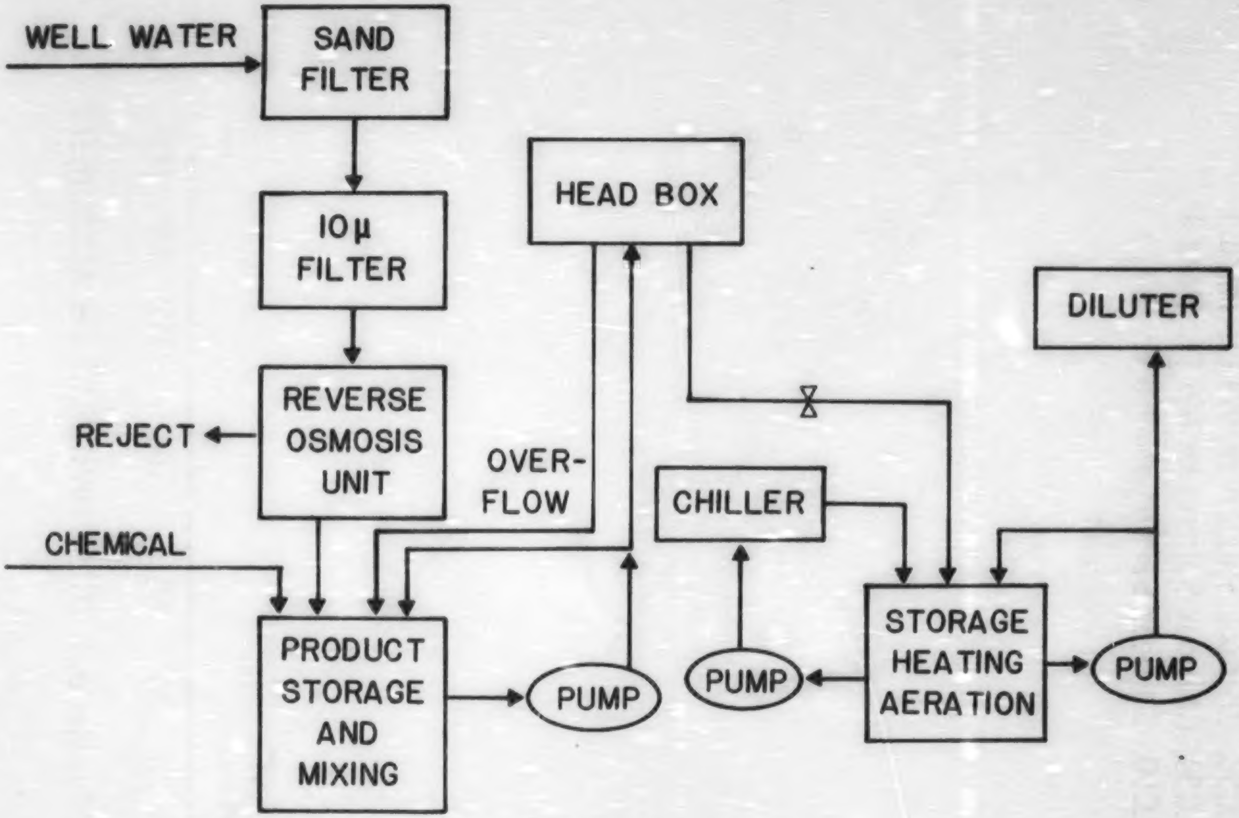
*Mention of product does not constitute endorsement by the Environmental Protection Agency.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CHILLER
PUMP
STORAGE HEATING AERATION
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SCHEMATIC OF RECONSTITUTED FRESH WATER FLOW-THROUGH BIOASSAY SYSTEM

Figure 4. Flow diagram of the system used to supply reconstituted freshwater for copper toxicity tests.

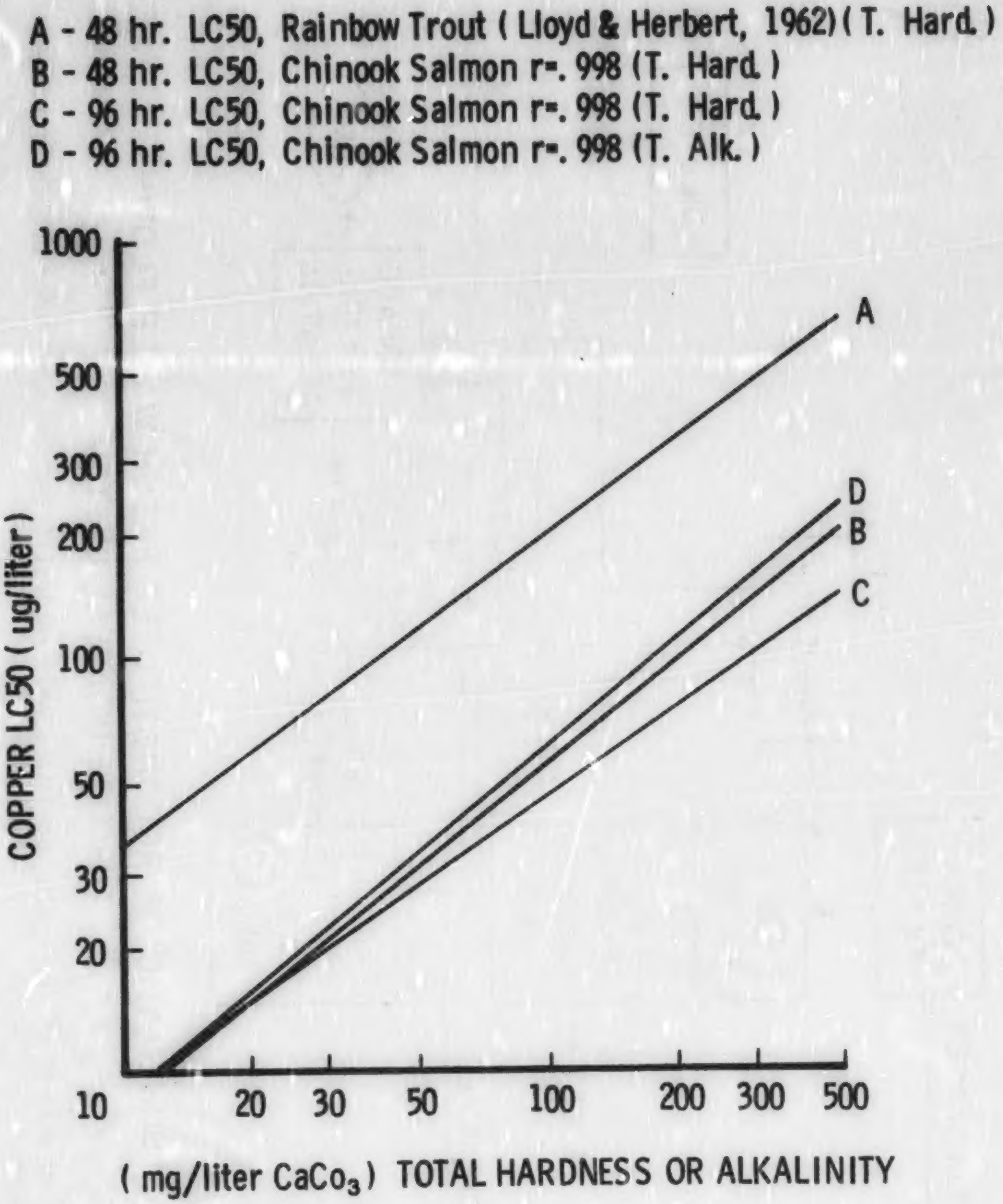

Figure 5. Comparison of relationships between hardness or alkalinity and acutely lethal levels of copper to rainbow trout and chinook salmon.

toxicity relationship shown in both studies could be explained by the alkalinity dependent carbonate complexation of copper as suggested by Stiff (1971).

However the 48-hr LC50 value for a given alkalinity from our study was lower than that of Lloyd and Herbert by a factor of 3 to 4. The most reasonable explanations for this divergence between the two studies lies in three areas of difference: fish species and size, test methods, and water quality. Studies at our lab have shown that steelhead trout (the anadromous form of the rainbow trout studied by Lloyd and Herbert) are slightly more sensitive to copper than are chinook salmon, so fish species differences may not explain the data; however, the chinook slamon used in our studies may have been smaller than the trout used by Lloyd and Herbert. The bioassays of these authors were static, changed every 24 hours (R. Lloyd, personal communication), while ours were flow-through. In our experience, lower copper LC50 values are obtained in flow-through bioassays than in static bioassays. The presence of strong copper complexing capacity of the type described by Chau, Gachter, and Lum-Shue-Chan (1974) in the water used by Lloyd and Herbert cannot be discounted; this phenomenon could produce the effect of raising the copper LC50 values in just the manner noted. However, the difference in bioassay methods was probably the biggest contributor to the observed differences in LC50 values between the two studies. Regardless of the difference between the two studies, it appeared that Stiff's (1971) explanation of the effects of carbonate in modifying copper toxicity were tenable based on our studies with reconstituted water.

One aspect of the alkalinity effect proposed by Stiff was that free cupric ion was a primary toxic form. This conclusion was supported by equilibrium calculations from published copper toxicity data compiled by Pagenkopf, Russo, and Thurston (1974) although they determined that $CuOH^+$ might also be involved. Additional evidence was developed by Andrew (1976) who showed that *Daphnia* survival time was proportional to cupric ion activity (Figure 6). Based on these results it appeared that copper toxicity was directly related to cupric ion activity and could be due primarily to that form.

Andrew (1976) also showed that the 96-hr LC50 of cupric ion activity was nearly equal for fathead minnows (*Pimaphales promelas*) in tests conducted in two appreciably different waters (Figure 7). Thus, while 96-hr LC50 values for total copper were about 200 and 800 μg/ℓ, the cupric ion activity was only 0.70 and 0.55 M/ℓ respectively. An exciting aspect of these results was that a given free copper concentration might be determined to produce a given effect regardless of water quality and total copper concentration.

However, when we looked at our bioassay data with respect to cupric ion activity we found that this relationship did not occur. Indeed, we looked at the 96-hr LC50 values in five different ways in regard to copper form and found no simplifying result in any case (Figure 8).

Interestingly, while total copper 96-hr LC50 concentrations increased with increasing alkalinity, cupric ion 96-hr LC50 values decreased with

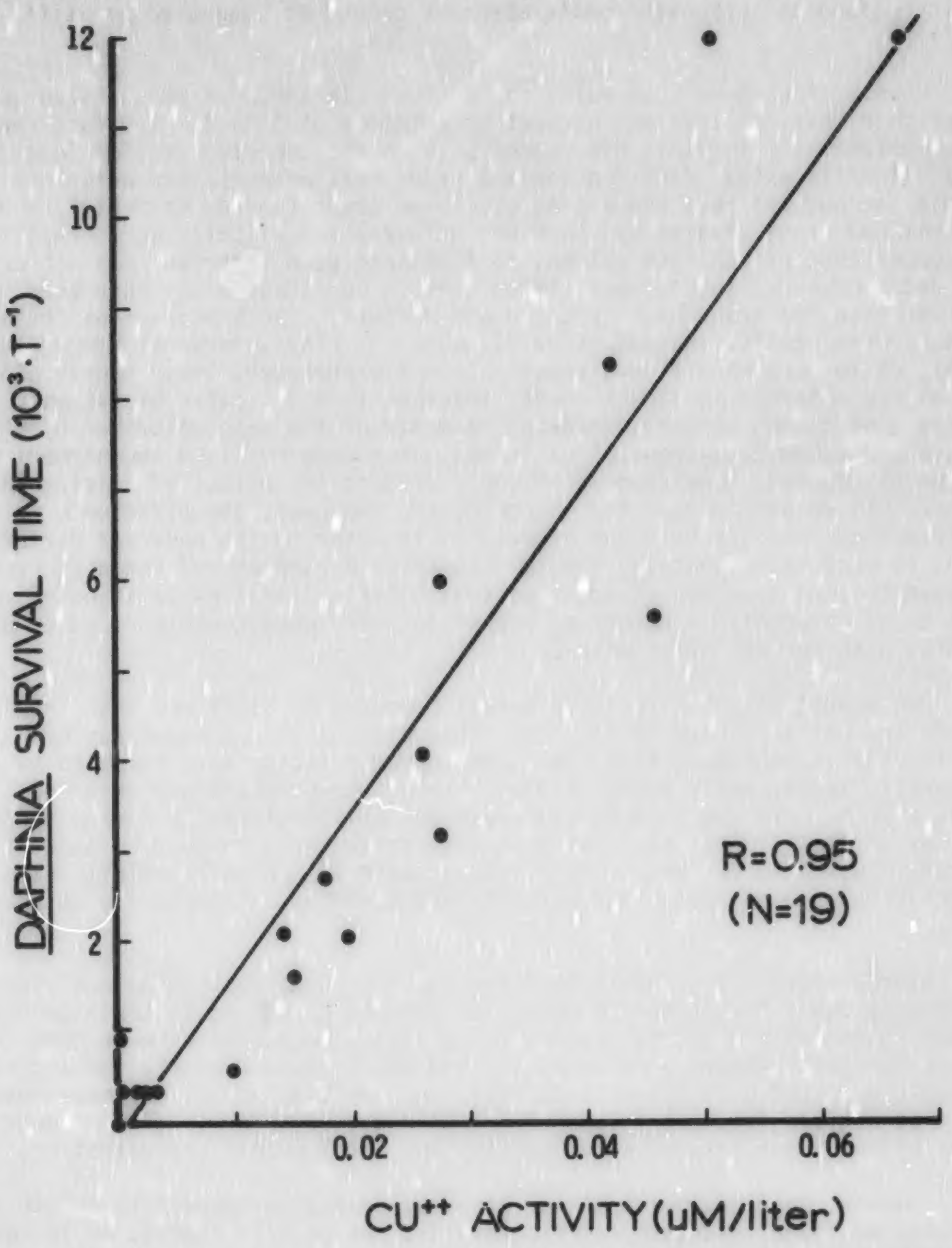

Figure 6. Relationhip of reciprocal survival time of *Daphnia magna* to cupric ion activity (Andrew, 1976).

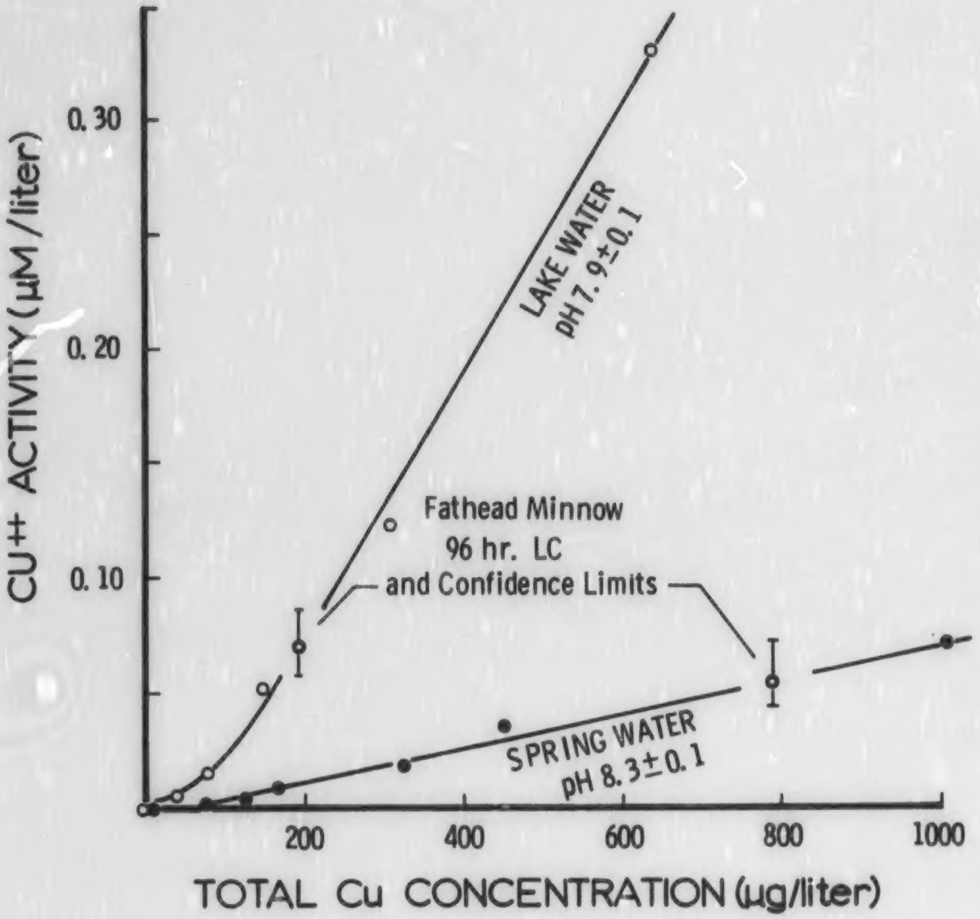

Figure 7. Relationship of cupric-ion activity, total copper concentration, and 96-hr LC50's for fathead minnows (Andrew, 1976).

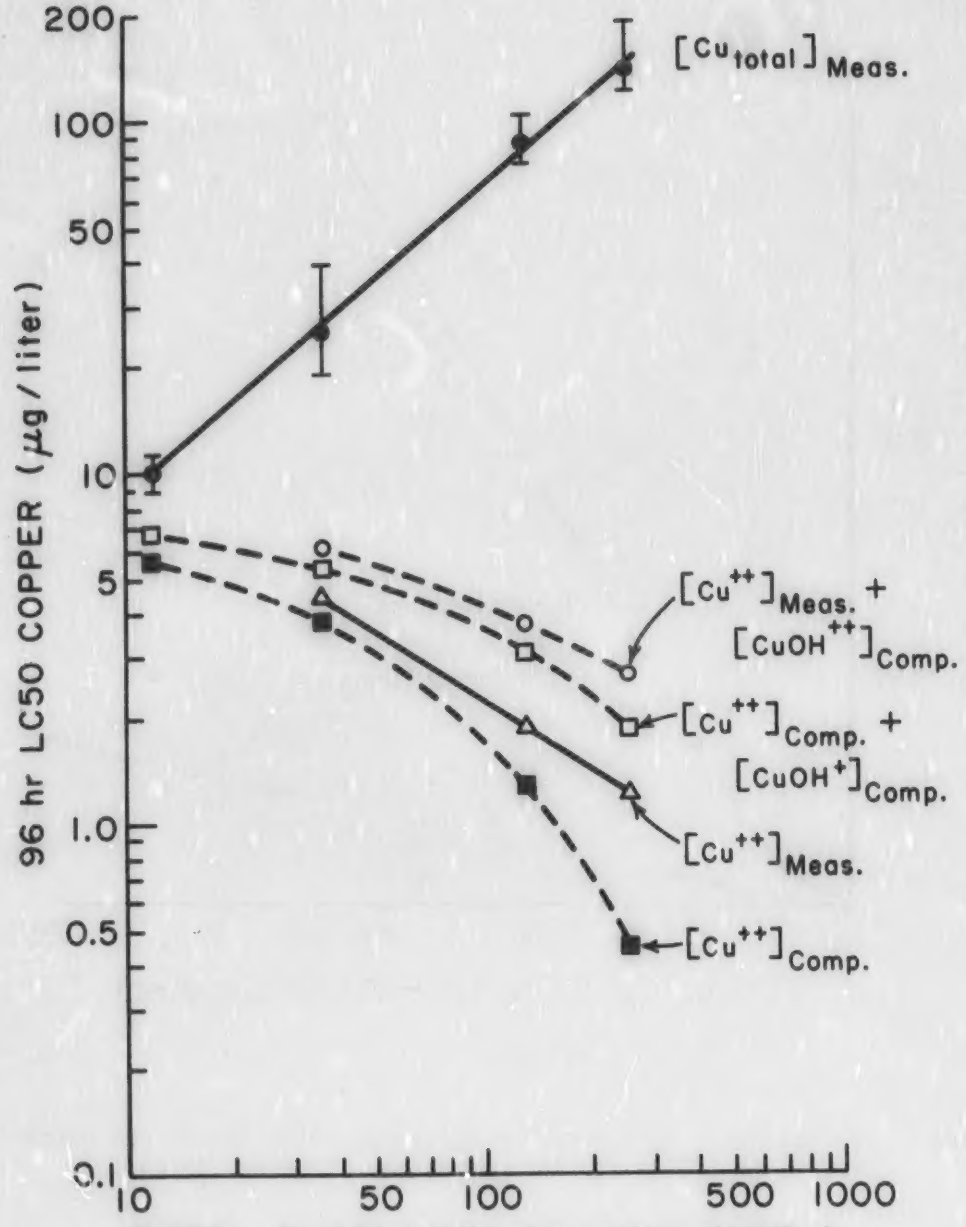

Figure 8. Relationship between alkalinity and copper 96-hr LC50 values for chinook salmon, expressed as measured total copper, measured cupric ion, theoretical cupric ion, and theoretical cupric hdroxide ion concentrations.

increasing alkalinity. Considerably more cupric ion activity was measured *in situ* (in the aquaria) than was predicted by the computer chemical equilibrium model. However, both the computer model and the cupric ion electrode yielded similar results when the electrode analyses were made on a sample gently stirred in a beaker. We presumed that the difference between aquaria and beaker determinations reflected higher cupric ion levels in the aquaria due to the relatively short reaction time of the cupric ions with the complexors in the dilution water.*

The data obtained in our chinook bioassays and those reported by Andrew (1976) for fathead minnows appear to differ, in that Andrew's data could suggest a constancy of cupric ion LC50 values while ours do not support such a constancy. However, if pH is treated as a variable the data from the two studies become more similar (Figure 9). Andres (personal communication) has also found that apparent cupric ion toxicity increases with increasing pH.

This tentative analysis suggests that pH may be an important factor in copper toxicity in addition to its usual association with alkalinity and the effect of pH on copper complexation equilibria. In a search for a simplifying premise we now wonder if the acutely lethal level of copper for a given species of fish would be some constant cupric ion activity level for a given pH value.

If this pH-constant relationship held true, then it should be possible to determine how much total copper would be required to produce an acutely lethal level of cupric ion activity in a specific water. Individual samples of water could be titrated with copper and a titration curve established from which cupric ion activity could be determined for any total copper concentration. Sample titration curves for two reconstituted waters are shown in Figure 10. The water without EDTA yields a straight line, with complexation due essentially to reactions with carbonate and hydroxide. The nearly parallel line of the water with EDTA added indicates a similar complexing capacity but only after the EDTA has strongly complexed an equimolar (10^{-6} M/ℓ) amount of copper. The X intercept in this instance represents a strong copper complexing capacity of the type described by Chau, Gachter, and Lum-Shue-Chan (1974). As would be expected there is essentially no strong complexing capacity in the reconstituted soft water.

We have run few copper titration curves for natural waters, but we are currently conducting a study to determine the copper complexing capacity of a variety of regional water. Initial results for two Oregon rivers are shown in Figure 11. We found that these titration curves did not yield straight lines, a result which we presume is due to the presence of multiple complexors at differing concentrations. Both river waters had a strong copper complexing capacity of <20 μg of copper/liter, and beyond the strong complexation, one river water sample (Alsea) had about twice the copper complexing capacity as the other (Willamette).

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*Recent experiments indicate no significant changes in cupric ion activity when aquarium 50 percent volume replacement time was increased from 30 min to 5 hrs.

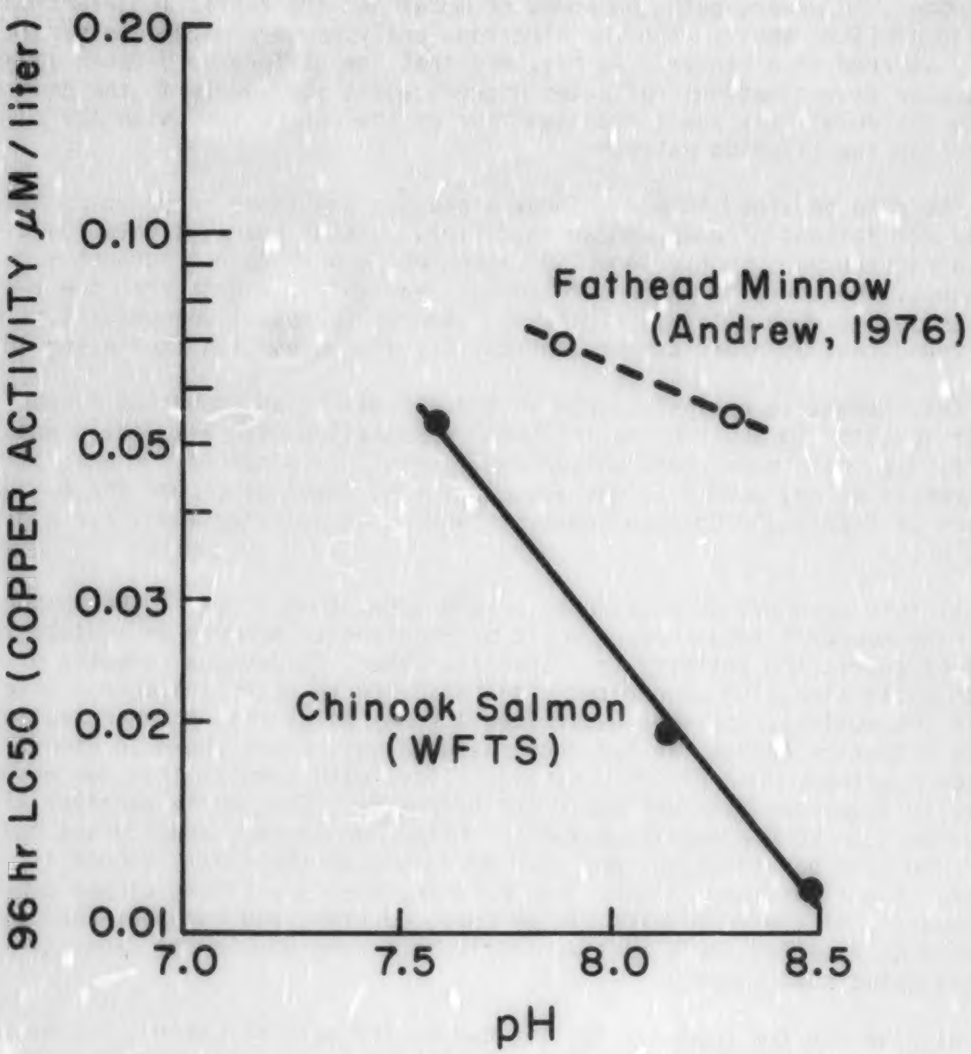

Figure 9. Relationship between pH and the 96-hr LC50 values of cupric ion activity for chinook salmon and fathead minnow.

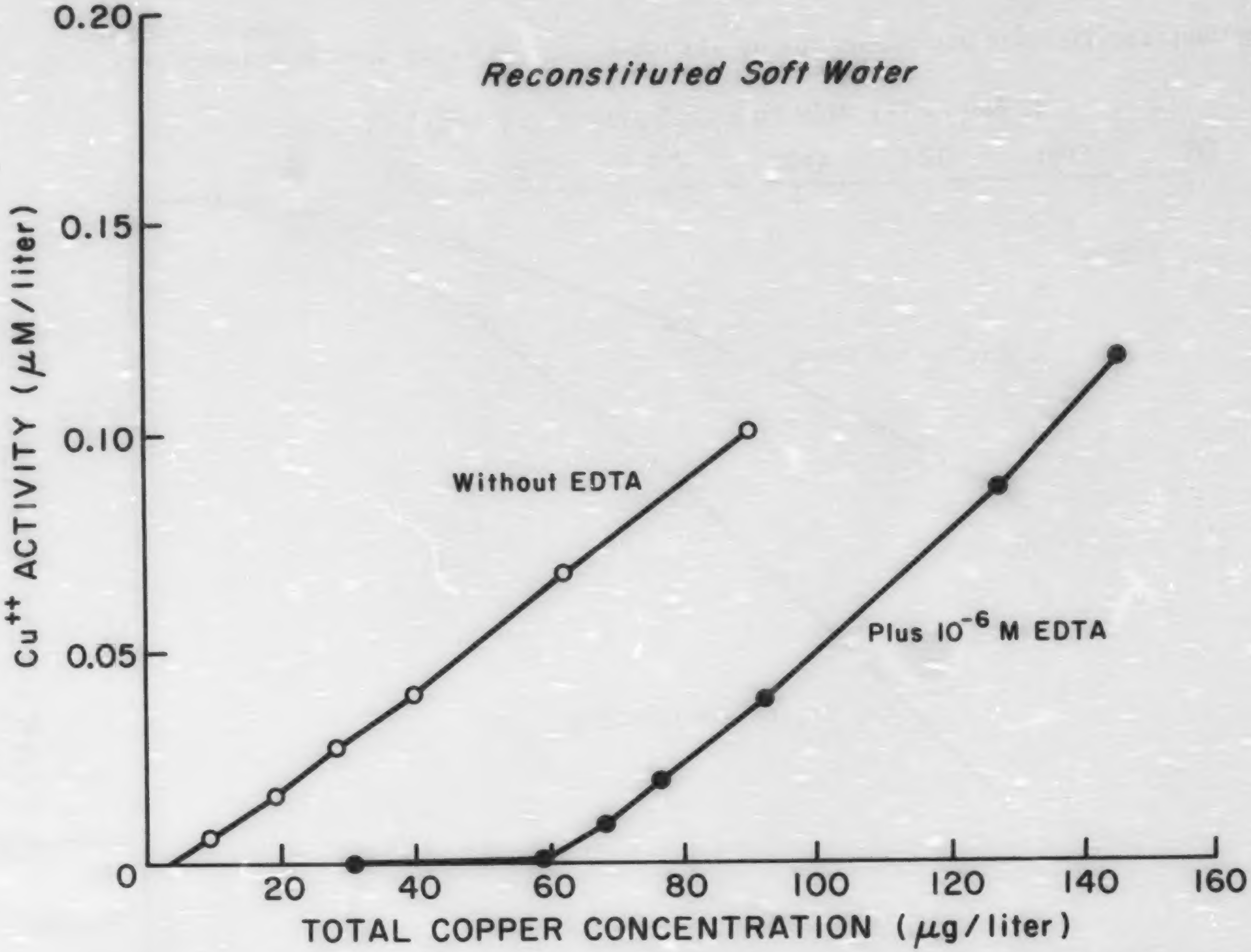

Figure 10. Relationships between total copper concentration and cupric ion activity obtained with reconstituted freshwater with and without EDTA.

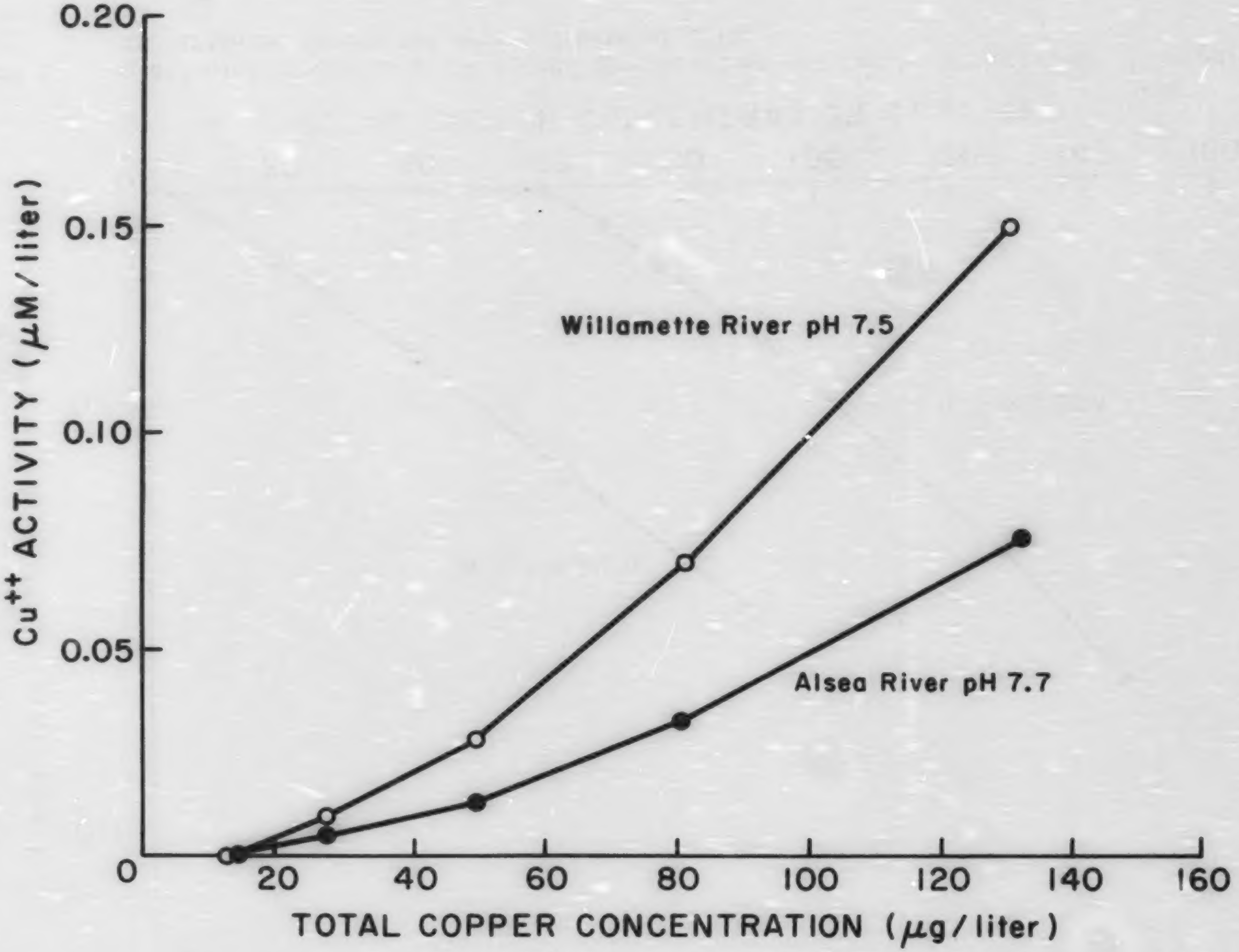

Figure 11. Relationships between total copper concentration and cupric ion activity obtained with two natural waters.

Returning to the copper activity 96-hr LC50 vs. pH relationship (Figure 9) we find for pH 7.5 and 7.7 corresponding LC50 values of 0.06 and 0.05 μM copper activity/ℓ. Utilizing these copper activity values and the titration curves shown in Figure 11, yields estimated 96-hr LC50 values for total copper of about 70 μg/ℓ for the Willamette River and 100 μg/ℓ for the Alsea River. If this procedure should prove tenable one could estimate lethal levels of copper for various waters by chemical means rather than by biological means. In some instances use of such chemical procedures could be highly advantageous. Regardless of its direct applicability, the knowledge about the relationships between receiving water quality and pollutant toxicity can aid in understanding the variability observed in studies related to fish toxicology.

I would like to conclude by placing the matter of copper form in a more general perspective. First, even if acutely lethal copper levels can be determined on the basis of cupric ion activity, a similar relationship with chronic toxicity is not assured. Therefore field studies, chronic toxicity studies, application factors, or short-cut indicator tests (e.g. the ventilation cough response) would still be required to estimate safe levels of copper. (It would be instructive to measure cough response and cupric ion activity in several freshwater matrices to see what relationships occur.) Second, based on the differences on copper activity observed *in situ* in the aquaria and in samples equilibrated in beakers such factors as pH, the copper form in the waste, and the reaction time in rivers or test aquaria are important determinants of cupric ion activity and presumably of copper toxicity. Finally, the data relating copper toxicity to cupric ion activity are far from being definitive. Nevertheless, this area of research promises to add appreciably to the understanding and predictive capabilities with regard to copper toxicity.

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THE ROLE OF CYANIDE AS AN ECOLOGICAL STRESSING FACTOR TO FISH

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ABSTRACT

Cyanide, at concentrations as low as 0.01 mg l^{-1} HCN, produces individual stresses on fish which, when integrated into a single total response, so seriously affect the energy supply processes, that both the range and scope for activity are reduced. The proposed toxicological model suggests that, at least under laboratory conditions, the fish could not continue to exist as populations.

This conclusion was drawn after evaluating the discrete effects of chronic cyanide poisoning on various fish tested under laboratory conditions in flow-through aquaria. The physiological responses tested were: embryological development; growth (wet, dry and fat weights) where a fixed or variable food ration, swimming velocity and initial size of the fish were tested; respiration and swimming after the poisoning period; histopathology of the liver; iono-and osmoregulation in varying salinities. Cyanide was also tested jointly with arsenic showing an additive deleterious effect on growth.

Not all physiological parameters were equally affected by cyanide but it appears that the greater energy-demanding processes, such as fat biosynthesis, osmoregulation and swimming were more seriously affected by this respiratory poison.

These results were integrated into a single eco-physiological response curve - a Relative Performance Index - which was used to develop a cyanide-stressed

Scope for Activity model. This model suggests a 50% reduction in the overall performance of the fish at 0.01 mg l^{-1} HCN and supports the previously established water quality criteria for cyanide, i.e. a maximum permissible level of 0.005 mg l^{-1} HCN.

INTRODUCTION

The continuous introduction of new chemicals into the aquatic environment poses a double challenge to the water pollution biologist:

1. He must measure indivudual responses of organisms to these new external stimuli at the physiological and/or biochemical levels keeping in mind the relationships to food, climate, niche and the animal community. Not only should these entities be recognized before testing, but their application should be within realistic limits such as food quality and quantity; temperature, flow and current of water; photoperiod; and, most important, the form under which toxicants are administered to the test animals.
2. He must evaluate the impact of the responses at the ecological level.

It is only in these terms that aquatic toxicology will produce new knowledge applicable to the definition of sound water quality criteria. It is essential to standardize the experimental conditions, thus requiring a laboratory approach to minimize the complex interaction of the multiple and uncontrollable environmental factors that prevail in nature. It is therefore the aim of the aquatic toxicologist to reach an ecological understanding of toxicants so that test organisms are exposed to realistic amounts and chemical species, for meaningful periods and through media (water, food, sediments, etc.) under which they occur in nature.

To relate aquatic toxicology to natural conditions, despite the unrealistic environment dictated by laboratory experimentation, one must first evaluate the relative importance of single physiological responses to the total performance of the animal in nature. This knowledge will come through simple concepts of animal activity such as growth, movement, reproduction, and behavior. Huntsman, (1948) defined the total response of animals to their environment as Biapocrisis. This conceptual approach had been elaborated by Fry (1947) who quantified ecophysiology with the concept of Scope for Activity, a measure under particular environmental conditions of the animal's metabolic energy available for activity above and beyond the minumum needs for maintenance (Figure 1). In a way, Scope for Activity is the total metabolic capacity an animal has available to meet the ecological realities of life in nature (Warren 1971, p. 148). Iverson and Guthrie (1969) have extended Fry's concept to natural populations integrating the total response of animals to environmental factors taken one at a time or interacting together. The "goodness of the habitat" which varies between upper and lower limits of environmental identities reflects the distribution and abundance of animals in nature from the center of distribution to the limit of their range (Figure 2). Iverson and Guthrie's most interesting contribution is the application of the notion of environmental stress to populations responding to natural and/or pollutional factors. The notion of stress must be taken positively as a response - sometimes useful, sometimes harmful - to the population. If

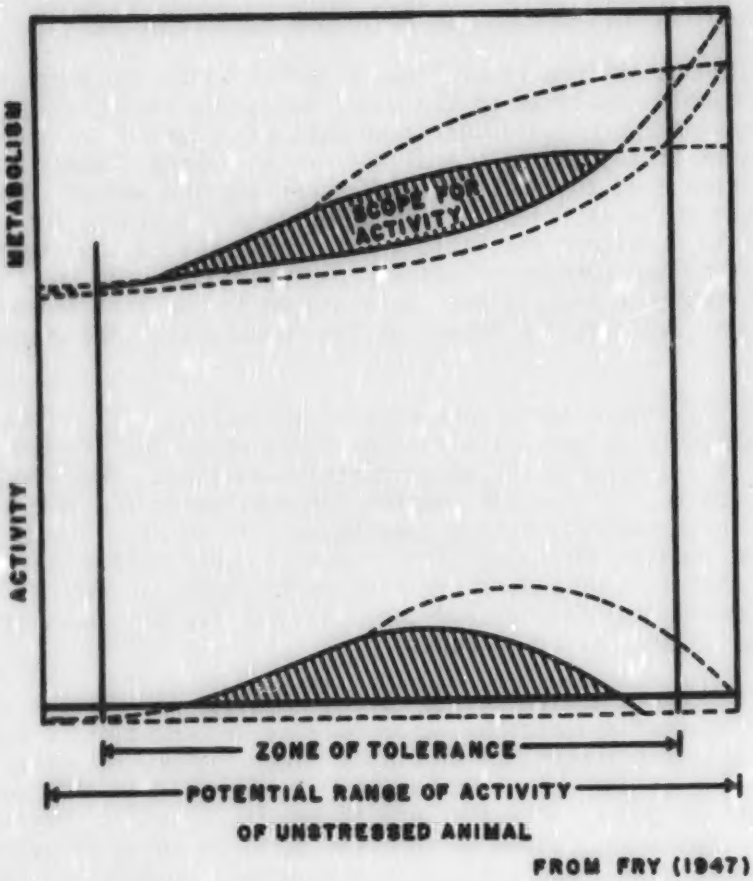
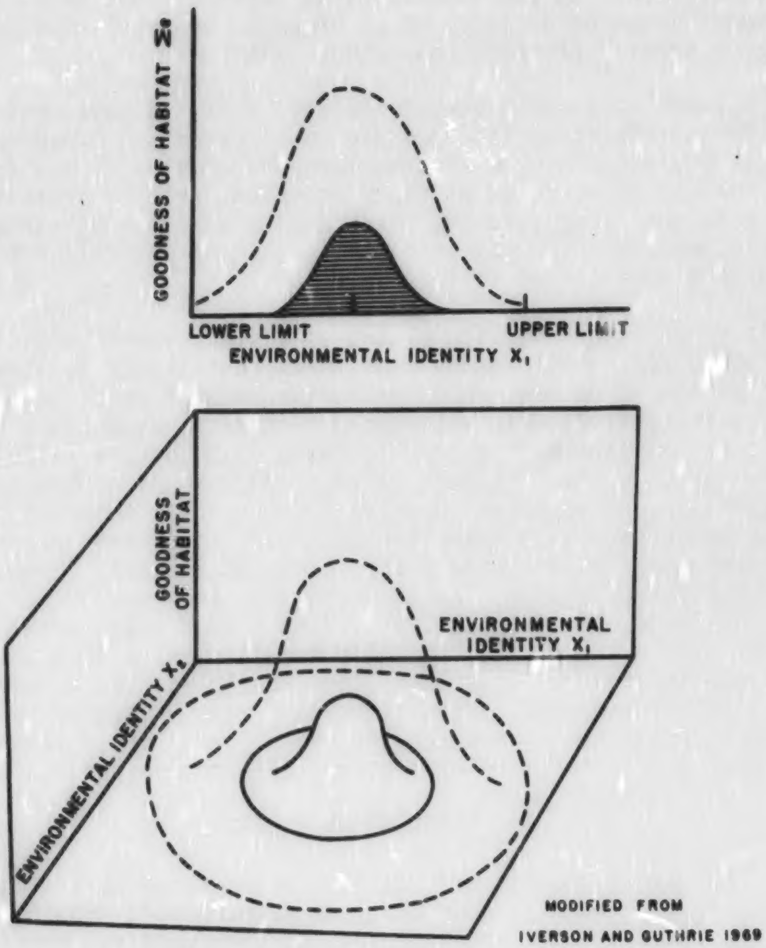

METABOLISM
SCOPE FOR ACTIVITY
ACTIVITY
ZONE OF TOLERANCE
POTENTIAL RANGE OF ACTIVITY
OF UNSTRESSED ANIMAL
FROM FRY (1947)

Figure 1. Diagram illustrating the standard and active metabolic rates of an organism subjected to an environmental factor, under normal conditions and under the influence of a stressing factor which reduces both the scope and range of the Scope for Activity. (Modified from Fry 1947.)


GOODNESS OF HABITAT Z_0
LOWER LIMIT
UPPER LIMIT
ENVIRONMENTAL IDENTITY X_1
GOODNESS OF HABITAT
ENVIRONMENTAL IDENTITY X_1
ENVIRONMENTAL IDENTITY X_2

MODIFIED FROM
IVERSON AND GUTHRIE 1969

Figure 2. Diagrams illustrating the response of a population: upper graph, to a single environmental entity under normal conditions (dotted line) and under the influence of a stressing factor (shaded area). In lower graph, same as above but for a population responding to two environmental entities without and under stress. (Modified from Iverson and Guthrie 1969.)

a population is reduced in size and distribution when responding to a natural factor(s), it is said to be under stress. This response might have a high selective value and be good. However, there are reduction levels from which a population could not recover and if, generation after generation, the population keeps shrinking, that stress is undesirable. "An environmental entity which is not lethal in the toxicological sense of that term, but affects the range and/or scope of activity of an organism or population is, ecologically speaking, a stress" (Iverson and Guthrie 1969).

We surmise that toxicants, in nature, do not always produce readily visible toxic effects on fish because of dilution and other masking factors. Ecological potential may be reduced through reaction at the individual physiological levels or stresses on other important related organisms, or both. By measuring in the laboratory the effects of a toxicant on various reactions of ecological importance it may be possible to model overall effects and postulate a safe application factor.

This paper is an overview of the effect of cyanide from the work of several co-workers who for many years have contributed to this subject. Cyanide, as simple molecular HCN or in the form of metal complexes, has been extensively studied in water pollution research and the literature of that subject has been extensively reviewed by Doudoroff (1976). Attention to cyanide as a research subject is valid because of double toxicological interest, basic and applied. Cyanide has long been known as a violent poison. Its properties as a selective inhibitor have been recognized as a useful research tool in respiratory physiology at the cell (Commoner 1940; Stannard and Horecker 1948; Keilin and King 1960) and organismal levels (Sumner and Doudoroff 1938) thus providing a good basic knowledge of its mode of action. As to its practical implications, cyanide is a common pollutant associated with mining. It is used in large quantities as a flotation reagent for silver and gold ores. It is also widely used as a complexing agent in the electroplating of zinc, copper and silver. In addition, the steel and chemical industries make wide use of this common chemical.

LABORATORY RESEARCH

Our laboratory studies with cyanide encompassed several aspects of the life cycle of fish namely, embryological development, growth, swimming, osmoregulation, respiration, histopathology of reproductive organs and liver. The test conditions varied in different experiments, but the most common experimental characteristics were as follows: all studies were conducted in the laboratory, with flow-through test tank systems supplied with dechlorinated water at pH of about 7.5, at temperatures of 10-25 C and for periods of 10-36 days. The test organisms were rainbow trout (*Salmo gairdneri*), a cichlid (*Cichlasoma bimaculatum*), Atlantic salmon eggs (*Salmo salar*), and coho salmon (*Oncorhynchus kisutch*).

EMBRYOLOGICAL DEVELOPMENT

The purpose of this study was to evaluate the impact of chronic cyanide poisoning on the early life stages of fish (Leduc 1977). Newly fertilized Atlantic salmon eggs were obtained from a hatchery and within 24 hours disposed into a series of test tanks, in renewed water and cyanide concentrations of 0.01, 0.02, 0.04, 0.08 and 0.10 mg 1^{-1} as HCN. The observations extended through hatching and up to the full resorption of the yolk sac of the control fry when the experiments were terminated. The eggs/fry were continuously exposed to cyanide during this whole period. The average temperature during incubation was 4.4 C; after hatching the temperature ranged from 3.5 to 8.3 C.

Cyanide markedly affected developing Atlantic salmon embryos. Hatching was delayed by three to six days and hatching success reduced by 20-40% in the range of concentrations tested (0.01 to 0.10 mg 1^{-1} HCN). During incubation, cyanide reduced the conversion efficiency of yolk into fish tissues so that at hatching, the cyanide-exposed fry were smaller in length and weight than the controls at all concentrations above 0.01 mg 1^{-1}. However, this impairment was rapidly overcome after hatching when the cyanide-exposed fry started to grow faster than the controls and, at the end of the experiment, they were all bigger than, or equal to the controls. Accelerated growth following a previous depression by cyanide has been observed by Leduc (1966) in juvenile cichlids and coho salmon and in juvenile rainbow trout (Dixon 1975; Speyer 1975). This phenomenon, which has not yet been explained, may be of physiological interest but of little ecological significance to the fry if other more serious effects of cyanide occurred during exposure. Indeed, we noted that many cyanide-exposed fry, although alive, were abnormal with gross deformities of the head, eyes, mouth and the vertebral column (Figure 3), anomalies that would be lethal in nature. The incidence of these macroscopic congenital defects ranged from 6% at 0.01 mg 1^{-1} to 19% at 0.10; the controls had less than 1% anomalies. To illustrate the overall effects of cyanide on the early life stages of Atlantic salmon a "realized viability" index was calculated by adding the values of percent hatching, fry survival and of "normal" fry. It appears from Figure 4 that cyanide reduced the "realized viability" at the lowest concentration, 0.01 mg 1^{-1}, by a significant amount and we believe that a much higher incidence of abnormalities would have been observed had histological techniques been used.

GROWTH IN RESPONSE TO CYANIDE POISONING

Cyanide, as a respiratory poison, would be expected to reduce the energy potential for growth in a way somewhat similar to the effects of low dissolved oxygen in the water (Warren, Doudoroff and Shumway 1973) by reducing food intake, conversion efficiency and/or biosynthesis. The study of the effects of cyanide on growth was introduced by Leduc (1966) working with a cichlid (Cichlasoma bimaculatum) fed unlimited rations of live tubificid worms. The cichlids were held in rectangular troughs, supplied with spring water heated to 25 C and subjected to various cyanide concentrations ranging from 0.01 to 0.10 mg 1^{-1} HCN for 36 days. Growth was measured as changes in wet weight.

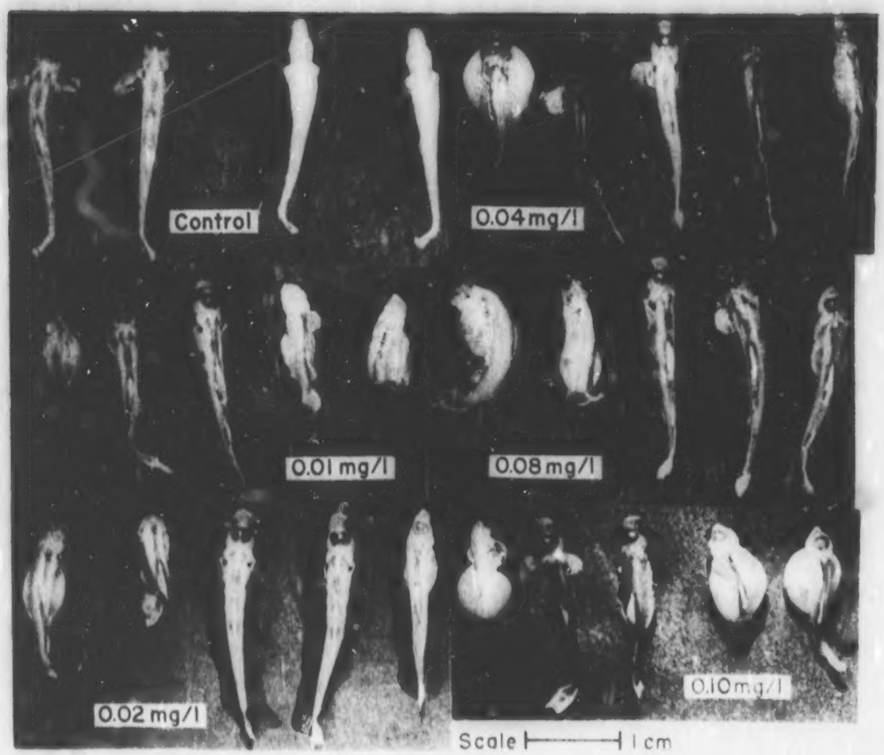

Control
0.04 mg/l
0.01 mg/l
0.08 mg/l
0.02 mg/l
0.10 mg/l
Scale 1 cm

Figure 3. Photograph showing typical body anomalies caused by continuous exposure of Atlantic salmon eggs and fry to sublethal concentrations of cyanide.

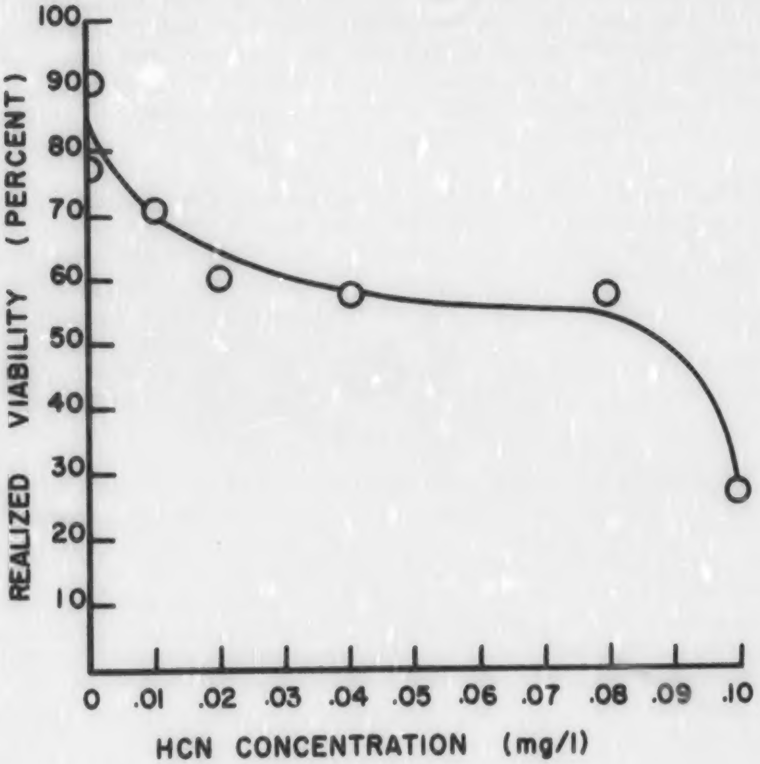


Figure 4. Realized viability of Atlantic salmon fry at different cyanide concentrations to which eggs and fry were exposed throughout development. (See text for details.)

Cyanide had different effects on growing cichlids, effects varying with time of exposure and with the concentrations tested. Cyanide promoted a higher food consumption; food conversion efficiency was initially higher at low cyanide levels around 0.02 mg 1^{-1} but lower at higher concentrations. The resulting growth was then initially better at low cyanide levels but less than the controls at higher cyanide levels (Figure 5). This pattern however changed with time. The initial growth advantage at low cyanide was lost and the cichlids exposed to higher cyanide levels exhibited a marked increase in growth rate by the end of the experiments. In other words, the cyanide-exposed cichlids were making up for the initial growth reduction, the effects increasing with the cyanide concentration. By the end of the 36-day periods there were hardly any differences between the control and the cyanide-exposed cichlids except a little depression at 0.10 mg/l HCN (Figure 5). Similar results were obtained by Leduc (1966) with coho salmon fed unlimited ration of earthworms in a flow-through system at 16 C.

Further studies of the effects of cyanide on the growth of fish were pursued with another salmonid fish, rainbow trout, focusing attention on other experimental and growth parameters. The temperature was lower (11-12 C), an artificial diet was given at different limited rations, and the effect of holding conditions was tested by comparing the growth of rainbow trout with and without swimming requirements. One study also evaluated the combined effects of cyanide with arsenic. As to the growth parameters, in addition to the wet weight, special attention was given to dry and fat weight changes.

Dixon (1975) exposed young rainbow trout to 0.01, 0.02 and 0.03 mg 1^{-1} for two successive periods of 9 days at 12 C while feeding them at a ration of 1.5 and 2.0% of body weight. Cyanide had an initial drastic effect, causing an almost complete arrest of growth at 0.03 mg 1^{-1} (Figure 5), but again the growth of cyanide-exposed trout showed a marked rebound in the second 10-day period. However, this response was not sufficient to compensate for the initial depressive effect of cyanide. Exposure to cyanide for 18 days resulted in significant reductions of growth at 0.02 and 0.03 mg 1^{-1} HCN. Speyer (1975) reached similar conclusions with rainbow trout tested at 0.02 mg 1^{-1} HCN and at 11 C.

McCracken (unpublished research, Department of Biological Sciences, Concordia University) considered three important bioenergetic components of fish growth affected by cyanide: activity, food ration, and size of the fish. Using a series of annular growth chambers equipped with motor-driven paddle wheels to maintain a constant current (Kruzynski 1972), he measured the effects of cyanide at 0.01 mg 1^{-1} using different food rations on young rainbow trout swimming at 12 cm sec^{-1} at 10 C. The results shown in Figure 6 suggest a size-related response. Whereas the small fish (8g) showed no response to cyanide at the different feeding levels it appears that for the larger fish (18g) cyanide markedly impaired food utilization with increasing ration. It should be noted that the food maintenance requirements (zero growth) does not seem to have been affected by cyanide as was the case for methoxychlor which markedly increased food requirements of brook trout (Salvelinus fontinalis) tested under similar conditions (Oladimeji and Leduc 1975). On the other hand when 11g rainbow trout were simultaneously

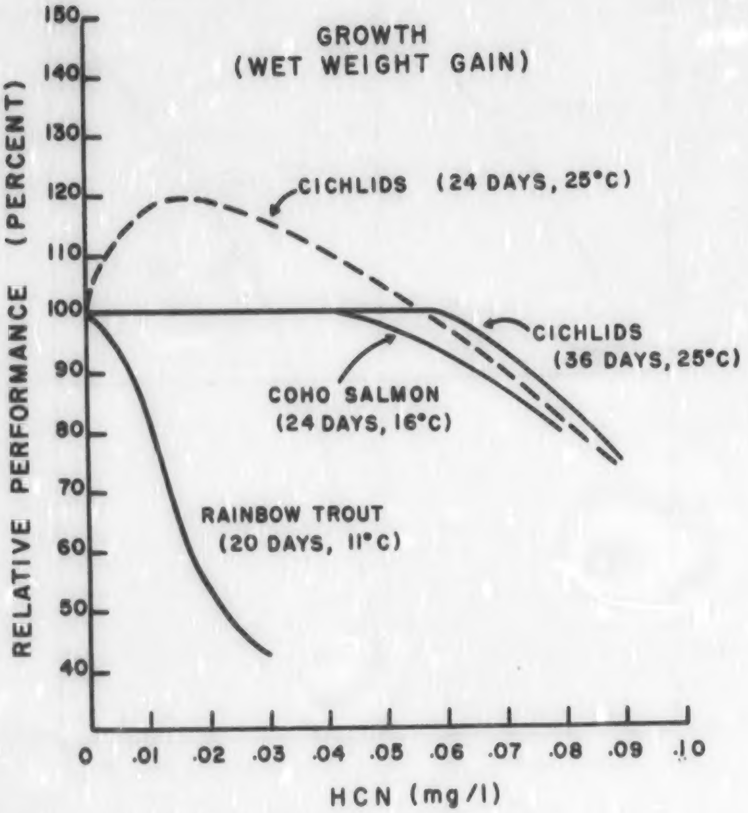


Figure 5. Relative growth index of various species of fish exposed to chronic cyanide poisoning throughout the experimental periods. The studies with cichlids and coho salmon were performed by Leduc (1966); rainbow trout were tested by Dixon (1975).

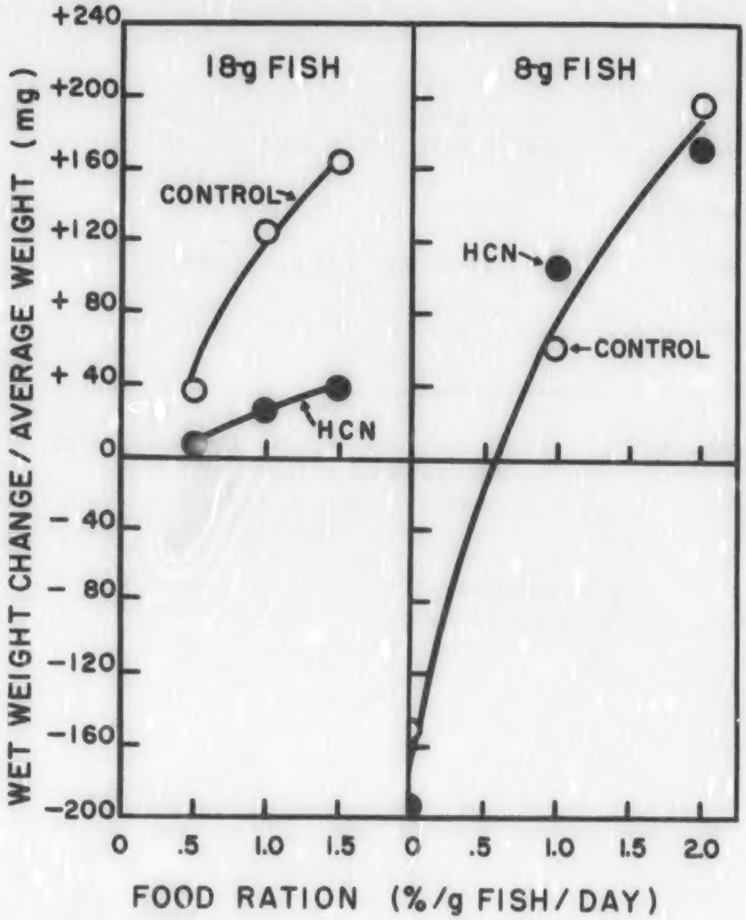


Figure 6. Comparison of the effects of various food rations during exposure to 0.01 mg l^{-1} HCN between two size groups of rainbow trout at 10 C. (From unpublished McCracken data.)

tested at 6, 12 and 20 cm sec^{-1} and fed one % of their body weight, McCracken (unpublished) found no significant effect of cyanide at 0.01 mg 1^{-1} (Figure 7). These results suggest that the differential effect of cyanide noted above is related to size more than activity.

The effect of size on the response of fish to toxicant has received some attention in the past, mainly at acutely toxic concentrations. It is noteworthy that Herbert and Merkens (1952) have clearly shown that the acute toxicity of potassium cyanide was greater to large rainbow trout than to smaller ones. On the other hand, Spear and Anderson (1975) found a reverse relation with the pumpkinseed sunfish (Lepomis gibbosus) exposed to acute levels of heavy metals.

In the natural environment fish are more likely to be exposed to a mixture of toxicants rather than single ones. In the vicinity of certain mines cyanide and arsenic occur together. Cyanide is used as a flotation reagent while arsenic leaches out of solid tailings from the oxidation of arsenopyrite. Speyer and Leduc (1975) found that exposure of rainbow trout to mixtures of arsenic and cyanide at the following concentrations: 0.02 mg 1^{-1} HCN and 3.0 mg 1^{-1} As; 0.02 mg 1^{-1} HCN and 6.0 mg 1^{-1} As, produced greater growth impairment than either arsenic or cyanide tested separately (Figure 8) the effects being additive following Finney's (1971) formula. These results also showed differential effects of cyanide and arsenic on wet, dry and fat weight gains (see Figure 8). This suggests on the one hand a greater water retention in the poisoned fish than in the control due to some osmoregulatory failure. Fat biosynthesis on the other hand, a high energy process, was obviously hard hit by cyanide poisoning as can be expected from a chemical acting directly on the respiratory-energy reaction chain. This phenomenon has been further demonstrated by Dixon (1975) and McCracken (unpublished).

The ecological implications of the disturbance of fat synthesis are important indeed to the survival of fish populations in nature where adequate fat reserves are essential for survival during adverse conditions and for yolk deposition in the maturing ovaries. The critical needs of fat deposits in yellow perch in nature were demonstrated by Newsome and Leduc (1975) and shown in Figure 9 which illustrates the seasonal changes of fat in sexually mature male and female yellow perch (Perca flavescens). During the fall, in the mature females, there is an important translocation of fat from the body to the ovaries which reduces the body fat content to a little over 2%, a level barely sufficient to sustain survival during the winter prior to spawning. This conversion is believed to be the cause of high winter female mortality which accounts for the low proportion of females (20%) in the populations of yellow perch inhabiting the cold mountain lakes of the Laurentians in which they were introduced about 30 years ago. It seems that these low productive lakes do not afford sufficient food for fat deposition to meet the maintenance and egg production by the females.

Looking at Figure 5 it would appear that there are specific differences of sensitivity to cyanide, cichlids being more resistant and rainbow trout the least. There are undoubtedly some specific differences but temperatures could have played a major role; cichlids were tested at 25 C., coho salmon at 16 C. and rainbow trout at 11 C. Recent findings by Kovacs (unpublished re-

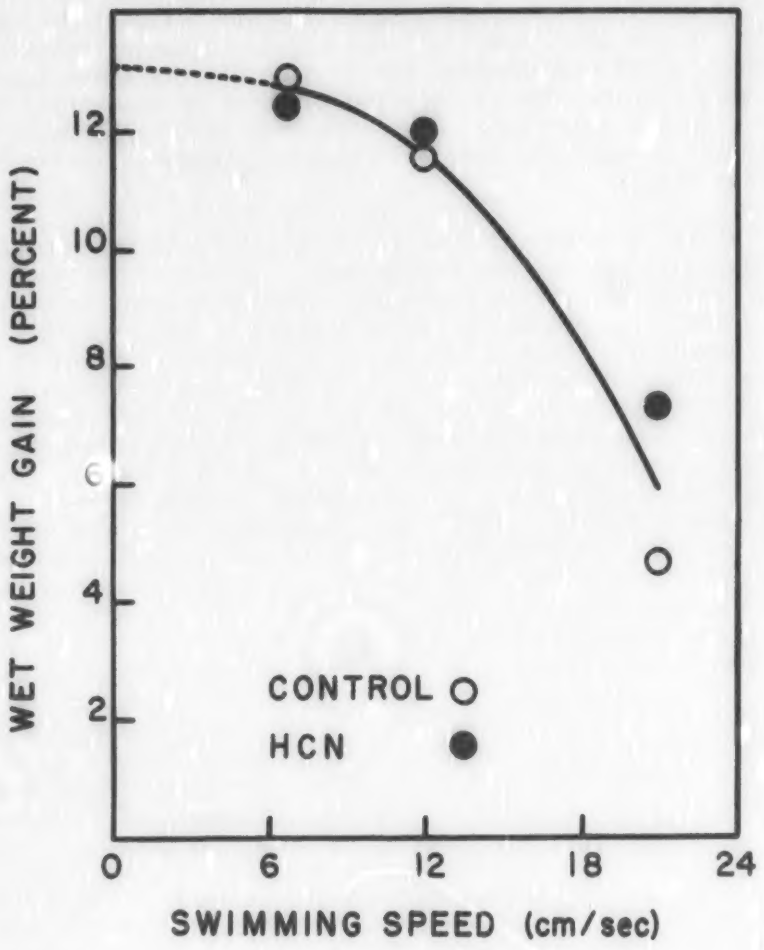


Figure 7. Relationship between the growth of control and cyanide-exposed rainbow trout (11.5g) held at different current velocities and at 10 C for 20 days. (From unpublished McCracken data.)

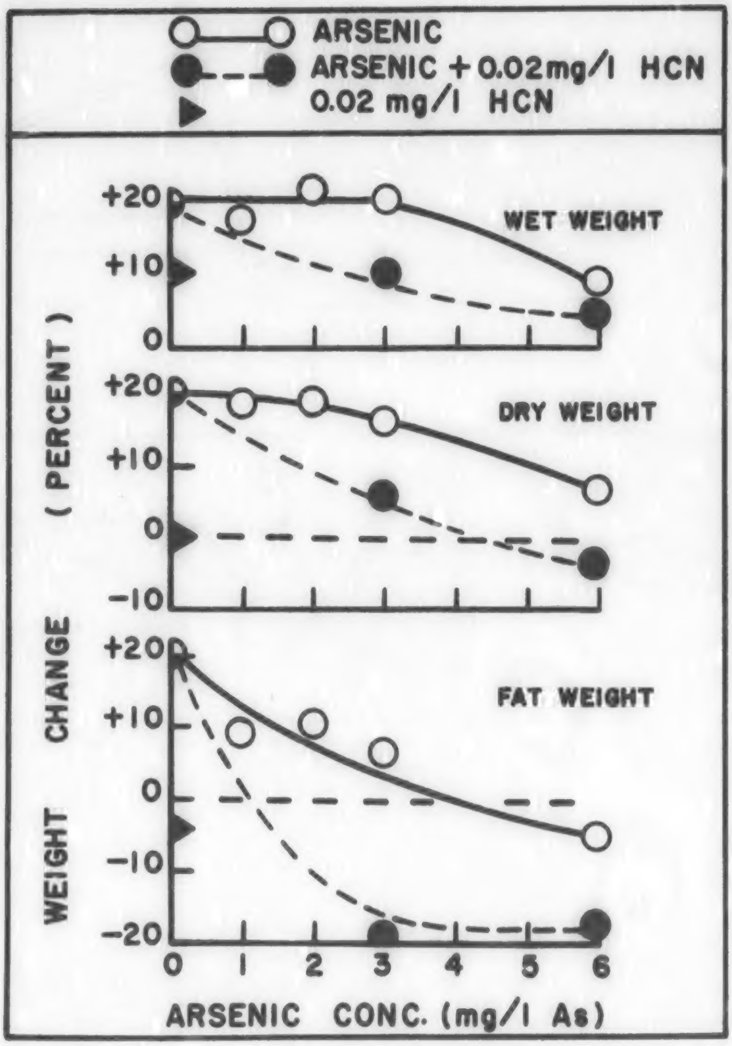


Figure 8. Effects of arsenic and cyanide, singly or in combination on the wet, dry and fat gains of rainbow trout after 21 days and at 11 C. (From Speyer and Leduc 1975.)

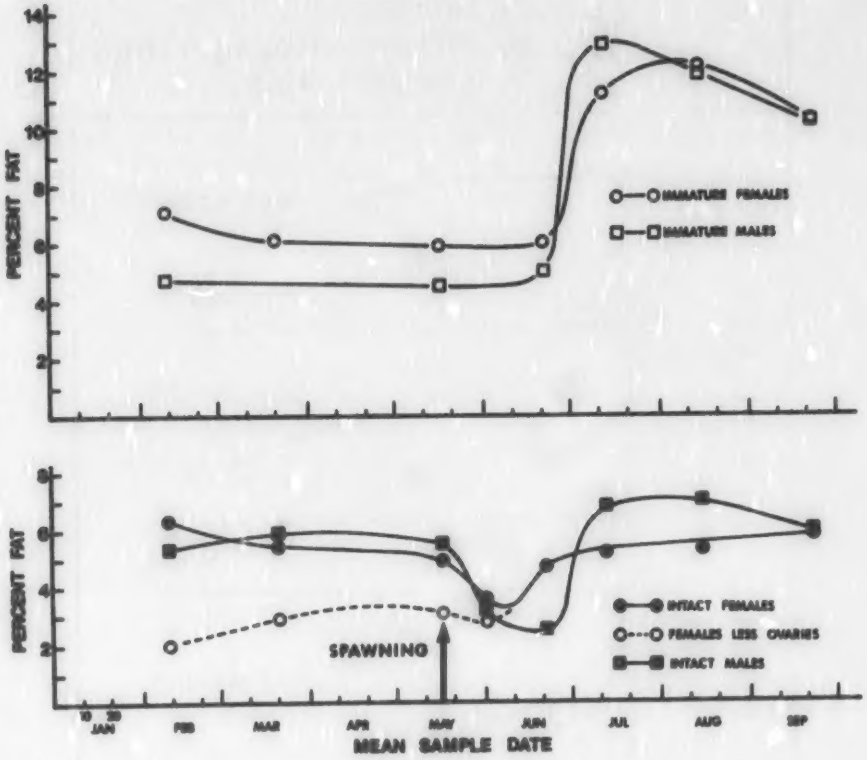


Figure 9. Seasonal fat content in immature and mature yellow perch in a Laurential lake. (From Newsome and Leduc 1975.)

search, Department of Biological Sciences, Concordia University) have now confirmed that cyanide is more toxic at low temperatures than at higher ones, both at the acute and sublethal levels. Tests were carried out with juvenile rainbow trout at 6, 12, and 18 C.

SWIMMING ABILITY

One effective way to evaluate the effects of environmental factors on fish activity is to measure their swimming ability. This response certainly has important ecological implications considering migration, maintaining position in a current or movements required in predator-prey interaction. Compared to growth, swimming requires a rapid mobilization of energy reserves and therefore relies for its performance on the well functioning of organs and intermediary metabolism. It is under stress, such as during swimming, that the overall fitness of an animal can be better evaluated than under the relatively passive conditions that exist when fish growth is measured in a tank, free of any rigorous swimming requirements.

Under low swimming velocities tested at 6, 12, and 20 cm sec^{-1}, McCracken (unpublished) found no effect of cyanide (0.01 mg 1^{-1} on the growth of rainbow trout fed at 1.0% of their body weight at 10.0 C. However, cyanide had a profound effect on the swimming ability of fish tested at higher velocities. Various studies summarized in Figure 10 illustrate the great sensitivity of fish to chronic cyanide poisoning. Cichlids were tested at 33.0 cm sec^{-1} and 25 C by Leduc (1966), rainbow trout at 47.0 cm sec^{-1} and 11 C by Speyer (1975), coho salmon at 48.8 cm sec^{-1} and 15 C by Broderius (1970) and brook trout at 55.8 cm sec^{-1} and 8 C by Neil (1957). As noted earlier for growth, the striking differences of swimming results cannot be explained as reflecting simply the difference in cyanide tolerance of the salmonids on the one hand and the cichlids on the other. The lower temperatures at which the salmonids were exposed during cyanide poisoning probably account for much of the apparent greater sensitivity of the salmonids.

It is also important to note that resumption of normal swimming ability after return to clean water is a very slow process, taking 15 to 20 days (Neil 1957; Broderius 1970). These results are indicative of very serious metabolic impairment by cyanide, and of inhibition of the oxidative pathways responsible for the maintenance of swimming. However, since the inhibitory action of cyanide on cytochrome oxidase is reversible (Stannard and Horecker 1948) and since cyanide is a non-cumulative cytoplasmic poison (Hewitt and Nicholas 1963), one would expect a rapid recovery after removal from a toxic environment unless there had been structural damage caused to the fish by the toxicant.

RESPIRATION

Respiration rate is widely used in physiology as a biological parameter integrating the overall metabolic activity of an animal in response to specific environmental entities. With fish, fundamental knowledge was acquired and new physioloecological concepts arose from metabolic rate studies (Fry 1947). Changes at the metabolic- or organ-functioning levels, or both, are reflected by changes in oxygen consumption and can therefore be a useful

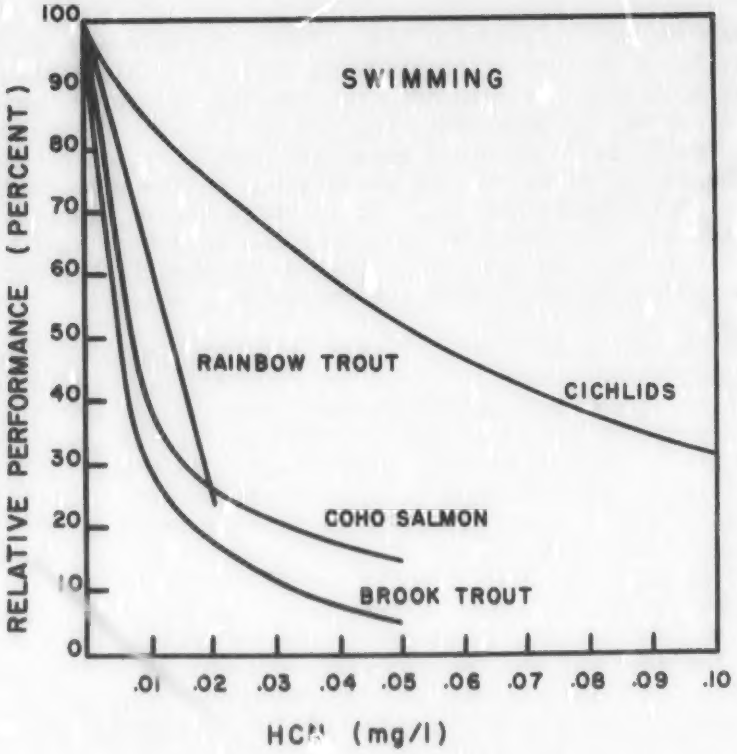


Figure 10. Effects of cyanide on the swimming endurance of various species of fish. (See text for details.)

diagnosis approach in fish toxicology. Indeed this proved to provide some explanation to the effects of chronic cyanide poisoning of fish when Dixon (1975) measured the resting metabolic rate of rainbow trout after exposure to the toxicant. After two 19-day growth experiments where groups of rainbow trout had been exposed to 0.0, 0.01, 0.02 and 0.03 mg l^{-1} HCN, individual trout were placed in black tube flow-through respirometers and the oxygen consumption measured for 6 consecutive days. The general pattern of the results is shown in Figure 11.

The metabolic rate of the controls progressively dropped probably as a result of quietening and starving. The cyanide-poisoned fish, however, first boosted their respiration, then stabilized it at a higher level than the control. We may speculate that the initial rise reflects a surge of oxidation of accumulated reduced metabolites upon return to clean water after 19 days of cyanide exposure. This stabilized higher metabolic rate may also be indicative of permanent damage by the previous exposure to cyanide that would reduce the metabolic efficiency of the fish or impose an extra metabolic load, both conditions resulting in an increased oxygen consumption. This increased cost of operation cannot but reduce the Scope of Activity (Figure 1) and may explain the very slow recovery of swimming performance of brook trout and coho salmon after previous exposure to chronic cyanide poisoning (Neil 1957; Broderius 1970). One has to look at some basic physiological functions before attempting any explanation of this increased metabolic rate. Osmoregulation appears to be promising, considering its bioenergetic and ecological implications.

OSMO- AND IONOREGULATION

Osmoregulation accounts for an important fraction of the basic metabolic rate of a freshwater fish which has to continually excrete an excess of water brought in by the osmotic gradient; this work also increases with activity. This phenomena has been well demonstrated by Rao (1968) who has calculated that osmoregulation may account for up to 20% of the active metabolic rate of rainbow trout. Ionoregulation also is a critical function which enables the fish to maintain the proper ionic strength in its tissues. The studies of osmo- and ionoregulation related to the action of toxicants are of double interest. They may provide highly informative clues to the overall performance of a poison while in freshwater and/or when salmonid smolts migrate to sea. Along these lines, Leduc and Chan (1975) have exposed rainbow trout to cyanide (0.01, 0.015, 0.021, 0.028 and 0.037 mg l^{-1} HCN) in renewed freshwater (10 C) for 28 days, then transferred them to artificial seawater (10 C). at 19.1 ppt but containing no cyanide; later the fish were returned to freshwater.

Cyanide affected both osmo- and ionoregulation in saltwater and in fresh water. Figure 12 shows that after 260 hours in saltwater the plasma chloride and plasma concentration were higher in cyanide-exposed fish, whereas upon return to freshwater (Figure 13) the reverse occurred, indicating loss of chloride and higher water content. In another test, we have shown that cyanide had an immediate effect on osmo- and ionoregulation when saltwater-adapted trout were transferred into freshwater-cyanide tanks (Figure 14). These changes may look small and of questionable ecophysiological significance but

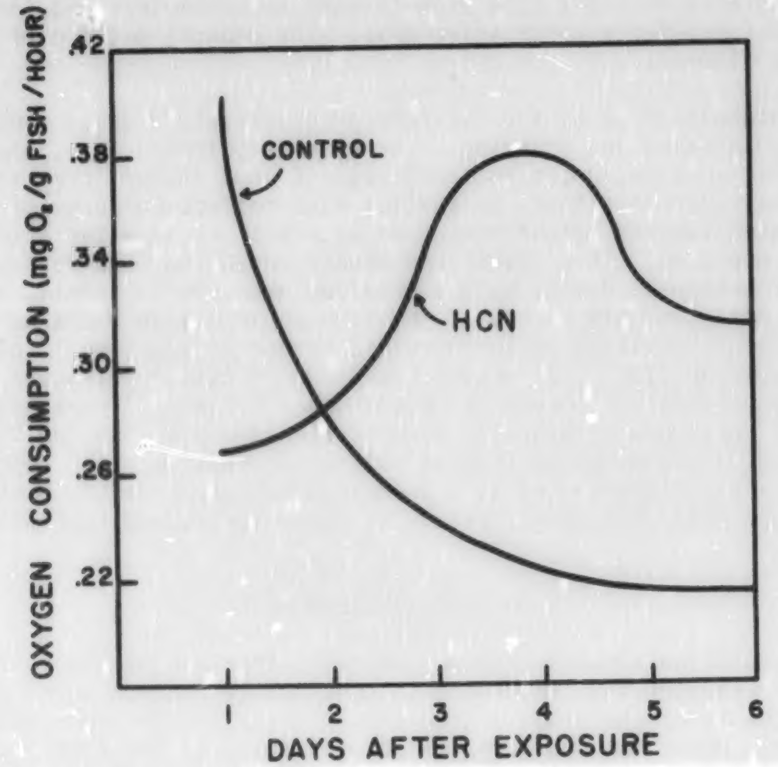


Figure 11. Generalized pattern of the resting metabolic rate in clean water of rainbow trout fingerlings comparing control fish to those which had been exposed to low cyanide concentrations (0.01-03 mg 1^{-1}) for 18 days at 11 C. (Modified from Dixon 1975.)

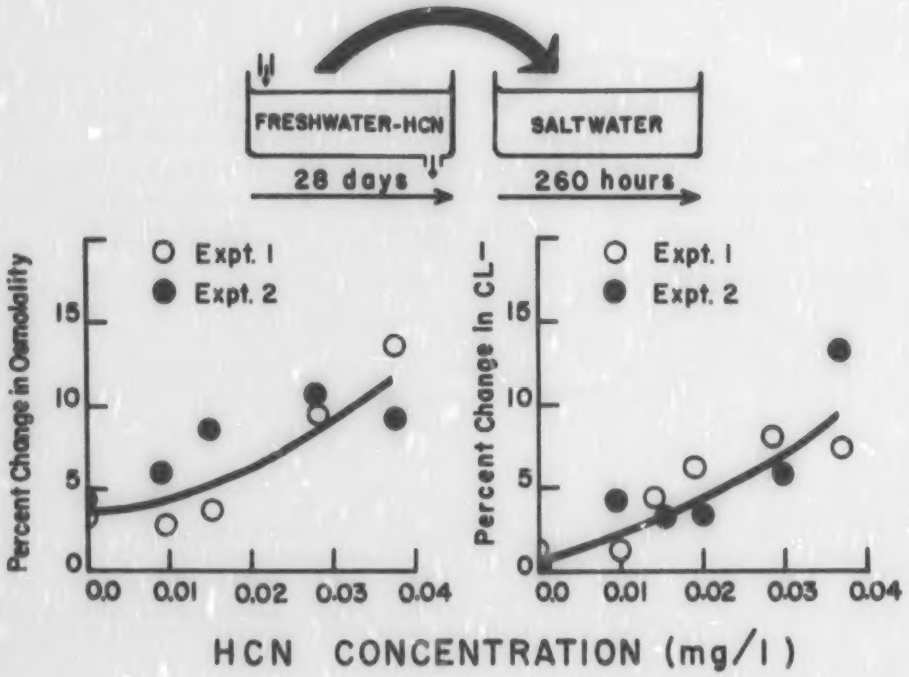


Figure 12. Relationships between the changes of plasma composition that occurred during a 260-hour salinity (18.9 ppt) tolerance test, and the cyanide concentrations to which juvenile rainbow trout had been exposed for 28 days in flow-through aquaria at 10 C. (From Leduc and Chan 1975.)

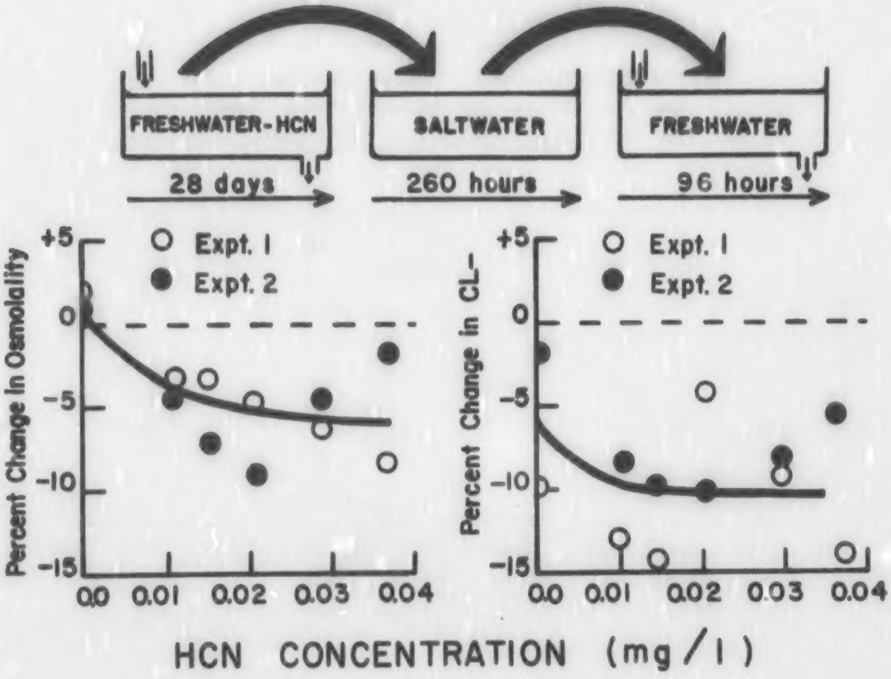


Figure 13. Relationships between the changes of plasma composition that occurred during fresh water exposure following transfer of the fish from salt water, and the cyanide concentrations to which juvenile rainbow trout had been exposed for 28 days in flow-through aquaria at 10 C. (From Leduc and Chan 1975.)

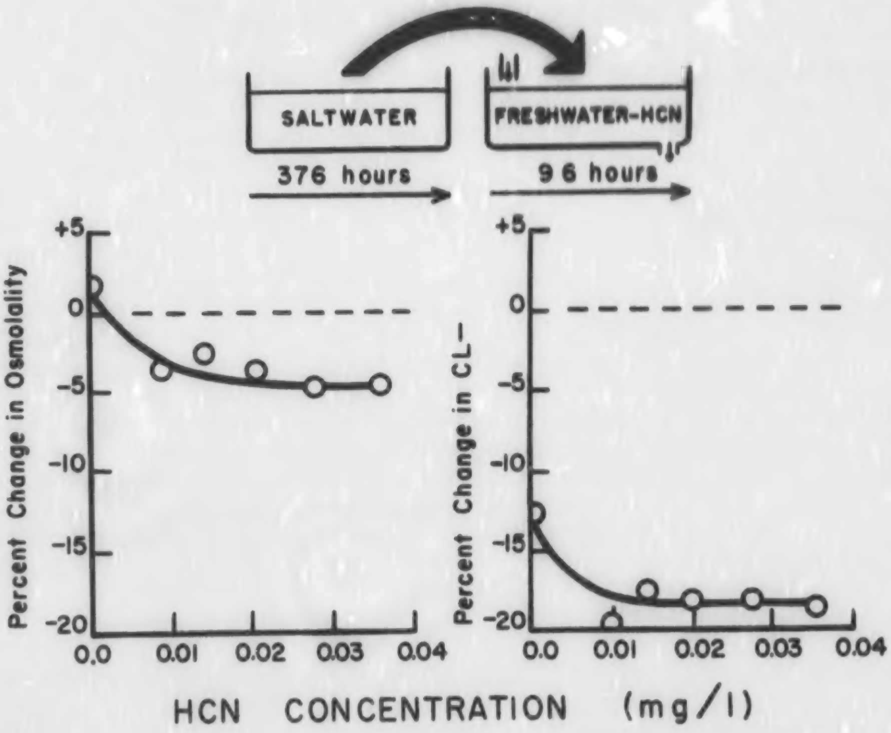


Figure 14. Relationships between the changes of plasma composition that occurred during a 4-day fresh water-cyanide exposure following transfer from salt water (18.9 ppt), and the cyanide concentrations to which juvenile rainbow trout were exposed in fresh water flow-through aquaria at 10 C. (From Leduc and Chan 1975.)

Wood and Randall (1973) have demonstrated that changes in water content as small as 1% correspond to marked increase in urine flow which is maintained by glomeruli filtration concommittant with high energy expenditures. It therefore appears that cyanide can have subtle effects measured in terms of water content in the fish but which are indicative of serious physiological impairment.

HISTOPATHOLOGICAL EFFECTS OF CYANIDE

Cichlids grown at 0.09 and 0.01 mg l^{-1} HCN not only had a markedly reduced swimming performance, but some showed serious body injuries at the end of the tests. Scales were falling off and short handling with a dip net was sufficient to severely damage the fin rays. Some also showed swelling of the body and extensive subcutaneous hemorrhaging. After the tests all fish were returned to clean water (no current) for observation and some died within 24 hours (Leduc 1966). No further examination was carried on these cichlids, but Dixon (1975) and Ruby and Dixon (1974) have shown histopathological effects of cyanide on rainbow trout. Dixon (1975) found that a 9-day period of exposure to 0.01 mg l^{-1} HCN was sufficient to induce extensive necrobiosis in the liver; gill tissue from the same fish showed no apparent cyanide-induced histopathological damage. Ruby and Dixon (1974) demonstrated blockage of mitosis in the testis of rainbow trout. Not only was the number of dividing spermatogonia reduced by a previous exposure to 0.01 mg l^{-1}, but of those cells that were dividing, mitosis was blocked in prophase with virtually no dividing spermatogonia reaching the later stages. Under these circumstances spermatogenesis would be completely arrested, preventing reproduction.

OVERALL SIGNIFICANCE AND CONCLUSION

We have shown that cyanide could markedly reduce the performance of several physiological functions of fish tested in the laboratory, but it is difficult to arrive at an overall significant judgement as to the toxicity of cyanide to fish unless one can sythesize various responses into one. Not all of the functions tested are of equal importance nor were they equally affected by cyanide. In an attempt to arrive at an overall evaluation of the chronic toxicity of cyanide, the approach taken by Warren, Doudoroff and Shumway (1973) was followed. It consists in drawing on the same graph experimentally obtained relative performance curves plotted against test concentrations while giving a value of 100 to the controls. Performance curves have been plotted in Figure 15 and a Relative Performance Index curve was then drawn, trying, to the best of our judgement to integrate in one line (heavy trait) a generalized response curve to cyanide. The Relative Performance Index curve suggests a 50% reduction of total performace at 0.01 mg/l, and further suggests that even though fish could survive indefinitely at 0.03 mg l^{-1} HCN in the laboratory, the different physiological requirements necessary to survive in nature could not be met.

The Relative Performance Index drawn out in Figure 15 was used in Figure 16 to model a Scope for Activity stressed by cyanide. The model shown in Figure 16 points out that, due to a marked reduction in active metabolism

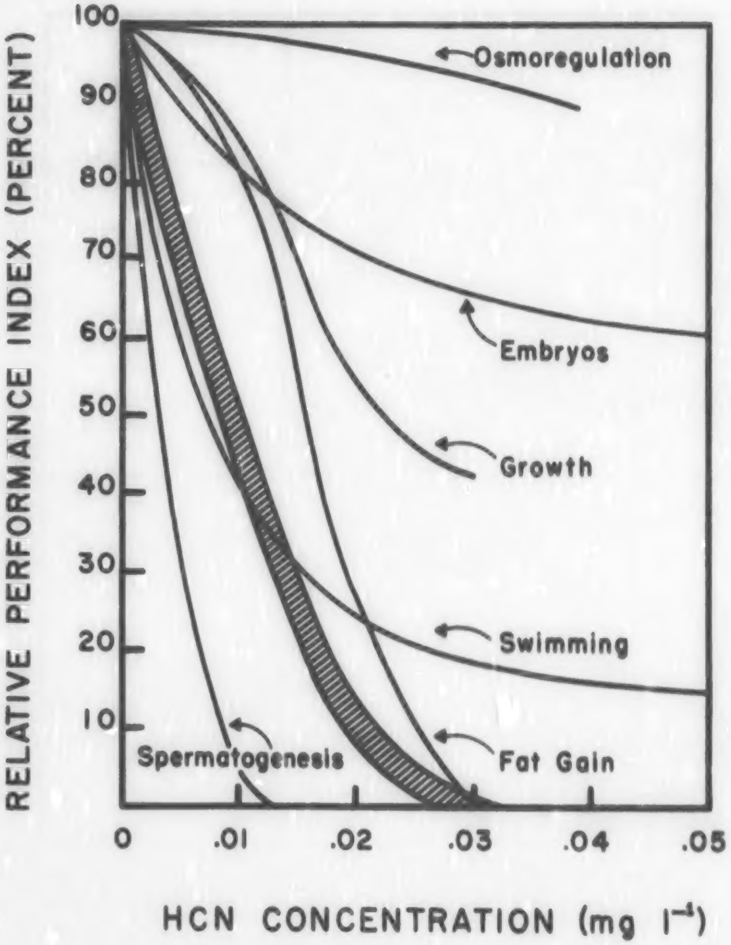


Figure 15. Relative performance of fish measured on various physiological responses to long term exposure to sublethal concentrations of cyanide. Details of the different experimental approaches are given in the text. The heavy line is a generalized estimate of the effects of cyanide on the overall performance of fish or a Relative Performance Index.

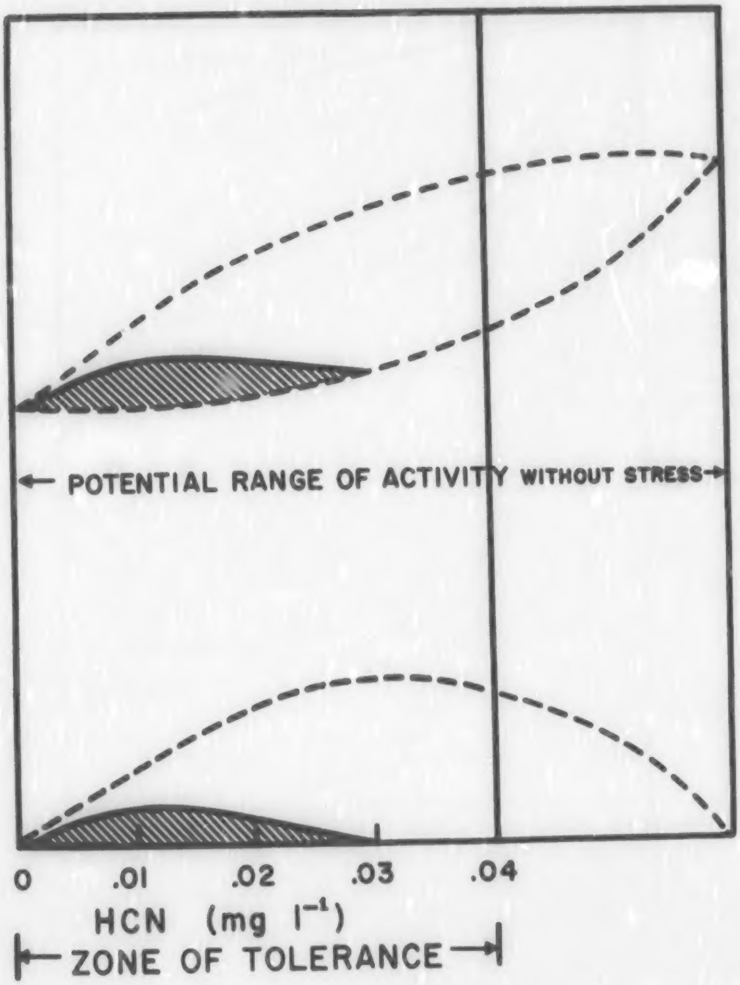


Figure 16. Comparison between a theoretical scope for activity of a fresh water fish without stress (open dotted area) and that under the effect of chronic cyanide poisoning at about 11 C (shaded area).

by cyanide, both the scope and range of activity were drastically reduced compared to that of an hypothetical unstressed fish.

This model was then applied to actual activity values published by Beamish (1964) who measured the routine scope for activity of brook trout at 5, 10, and 15 C. The model in Figure 17 shows that the routine scope for activity would be reduced to zero by 0.03 mg 1^{-1} HCN and by 50% at 0.01 mg 1^{-1}. There are, however, two additional points to consider. The measurements of the routine scope for activity of brook trout published by Beamish (1964) showed a maximum at 10 C. The optimal temperature varies with different species of fish but if these data are directly transposable to the natural environment, one would expect to find the brook trout most successful at 10 C whereas 5 and 15 C would curtail the abundance and distribution of this species. If, at these temperatures, (5 and 15 C) with safety factors lower than at 10 C, the population was stressed by a toxicant it would experience such a reduction in its capacity to reproduce that it could lead to the extinction of the population. With reference to cyanide, this effect would be even greater at 5 C than suggested on our model since, as mentioned before, cyanide, at low concentrations is more toxic at low temperature.

At present it is recommended that the level of cyanide in water never exceeds 0.005 mg 1^{-1} at any time or place (National Academy of Sciences and National Academy of Engineering 1972). According to our model, concentrations between 0.01 and 0.005 mg 1^{-1} HCN would reduce the fish performance or scope for activity by 30-50% (Figure 13). What reduction of scope can be accepted as an application factor remains a difficult and somewhat arbitrary decision to make. With regards to oxygen alone, Fry (1960) suggested that a 50% reduction could be a reasonable estimate of the oxygen requirements of fish in nature. This view has however not been supported by Doudoroff and Shumway (1970) and, to our knowledge, this question has been not considered with toxicants. If Fry's suggestion of accepting a 50% reduction of scope is taken, the recommended level of cyanide in water would fall within the values of 0.01 mg 1^{-1} proposed by Jones in 1964 and 0.005 mg 1^{-1} established by the Environmental Protection Agency (National Academy of Sciences and National Academy of Engineering 1972). There is however some reservation to the acceptance of these criteria.

Histopathological observations have revealed some very deleterious effects of cyanide at 0.01 mg 1^{-1}, a concentration that otherwise showed no marked effect on growth. Also, the blockage of spermatogenesis and, possibly, oogenesis could have dramatic effects leading to the disappearance of entire populations resembling the effects of acid pollution on lakes in northern Ontario (Beamish 1974).

Most water quality criteria developed for North America have undoubtedly been developed to protect waters of the temperate regions. If, as our recent studies suggest, cyanide chronic toxicity increases with decreasing temperature then the recommended levels may not be safe under cold climates such as in northern Canada and Alaska where cyanide is extensively used by the mining industry.

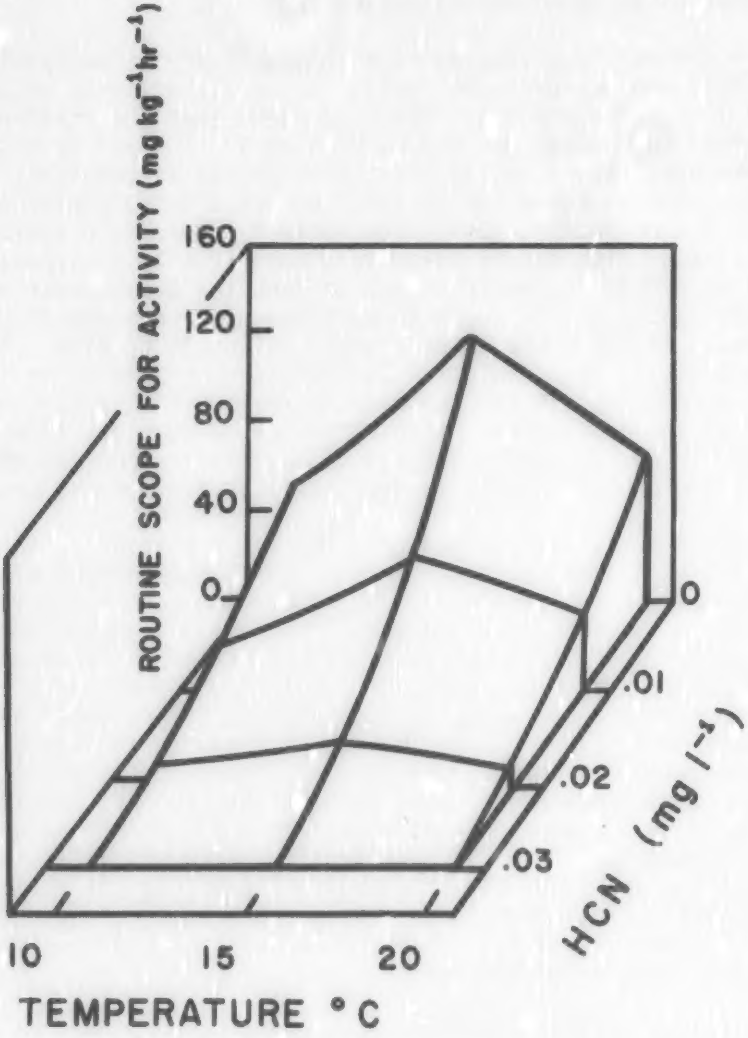


Figure 17. Estimate of the effects of cyanide on the routine scope for activity of a salmonid fish at different temperatures. This model was drawn by applying the Relative Performance Index values at different cyanide concentrations as determined from Figure 15 to an hypothetical salmonid fish using Beamish (1964) routine scope for activity data obtained on brook trout, *Salvelinus fontinalis*.

Finally, further studies of cyanide should concentrate at levels lower than 0.01 mg l^{-1} HCN. The lack of studies carried at lower concentrations is mainly due to the lack of a suitable method of detection below 5 ppb which is the lower limit of the techniques currently used.

ACKNOWLEDGEMENTS

The pursuit of scientific knowledge is a long, arduous, sometime obscure path, but the presence of leadership, inspiration and close collaboration along the way make achievement possible. This research on one common pollutant, carried over many years with the hope that some benefit to our environment will be derived, would not have materialized without many inspirational associations. I hereby wish to recognize the leadership of Dr. Peter Doudoroff and Dr. Charles E. Warren, of Oregon State University, and the generous assistance of George Chadwick at the beginning of these studies. May I also acknowledge the excellent collaboration of Dr. Sylvia M. Ruby, Associate Professor of Biology at Concordia University, and of graduate students, Ken S. Chan, D. George Dixon, Ian R. McCracken, George E. Newsome, Menno R. Speyer, Tibor G. Kovacs, Adebayo A. Oladimeji, Walter Banas, jr. and George M. Kruzynski.

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AN ASSESSMENT OF APPLICATION FACTORS IN AQUATIC TOXICOLOGY

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ABSTRACT

In the early 1950's, application factors to estimate "safe" concentrations from LC50's were proposed. Later, an experimental method of estimating the numerical value of the application factor was proposed to replace arbitrary values such as 1/10. Both measured values and arbitrary ones have been widely employed in water quality criteria by regulatory agencies. An examination of the data base for establishing application factors for various pollutants in different water types and for various species, reveals an unacceptable spread in their numerical value. Several factors such as chemical effects of the water on the pollutant, experimental error and biological variability must be contributing to this spread thereby making a determination of their real validity difficult. A better method to predict concentrations that will not affect survival, growth, and reproduction is needed for present toxicological requirements.

Aquatic toxicologists of today are faced with an enormous pressure to provide decisions regarding the potential effects of hundreds of chemicals should they be released into the environment. While everyone agrees that "more research is needed," we also must realize that urban and industrial development will proceed regardless of the need for more research, and therefore decisions must be made now. Some of us may find untenable the passage of laws before sufficient data are available to confidently make the needed decisions. As scientists we must agree that decisions or predictions based on skimpy data can still be scientific. As long as the basis of the decision and the proper confidence limits are provided, scientific integrity is maintained. Indeed, is not science in essence a process of "concluding from available data" that which can be concluded rather than requiring a fixed or predetermined amount of information.

It is in this framework that today's aquatic toxicologists must render "scientific judgments" to meet today's problems. Answers derived from available data, however scant, are better than pure guesses.

The Toxic Substances Control Act passed just prior to this writing, brings to the field of aquatic toxicology demands for decisions that far exceed any experienced heretofore. Under this new law, decisions on environmental as well as other effects must be made long before a proposed product is in use or introduced into the environment. Of necessity, then, regulatory decisions will have to be based on laboratory studies of a relatively few species and predictions made for many other species. Only after the early decisions have been made regarding acceptable concentrations will field data be obtained to verify predicted effects, because only then will the product be in use and extant in the environment to produce these effects. Against this background I want to discuss the present status of the application factor hypothesis (Mount and Stephan 1967) for use in predicting acceptable concentrations for aquatic organisms.

Before proceeding, I want to acknowledge the difficult and laborious effort of a special staff committee of the Environmental Research Laboratory in Duluth. This committee, composed of Robert Andrew, Duane Benoit, John Eaton, James McKim and Charles Stephan, now have in press their report entitled "An Evaluation of an Application Factor Hypothesis" for which they have sorted and assembled most of the toxicity data base pertinent to the validity of experimentally derived application factors. Without their report as source material, this paper could not have been presented at this time.

Let us first summarize the considerations that must be made when one predicts the acceptable concentrations for aquatic organisms. I will leave to others the difficult chore of extrapolating results from species to communities to ecosystems; instead I will focus my comments on predicting effects from test specimens to species in their normal niches.

Any prediction must consider whether the animals tested are typical of the species as a whole. Certainly one would not choose inbred or geographically isolated populations if the data are to be broadly applicable and are expected to account for extant species variability. Since one nearly always is concerned about protecting more than a single species, the difference in sensitivity between species also must be considered. The existing toxicological data adequately demonstrate substantial differences between species for many toxicants.

The physical and chemical changes brought about by the common components in surface waters and the resulting changes in the toxicant, were recognized early. Much early aquatic toxicological work involved metals and the effect of pH and hardness on their toxicity. So great were these effects that inclusion of "water hardness" effects on toxicity (even when there is no reason to expect effects) have been routinely included in subsequent experimental work. Even pollution control administrators who know little about the subject will raise this issue in the standard setting process. Unfortunately, I think we have failed to recognize the important role played by other components, for example, suspended solids. Clay or algal cells and chelating agents such as humic substances certainly must affect the toxicity of many contaminants, particularly the less water soluble synthetic organics.

The length of exposure time to the toxicant and the organism's life history stages likely to be exposed are other important considerations. Often one does not have chronic exposure data for many species when acceptable concentrations must be predicted. We recognize, however, that few or no generalizations can be made about the shape of time-effect curves beyond "acute" periods of exposure, thus leaving much uncertainty about the effect of exposure length on acceptable concentrations and no way to extrapolate effects for longer, untested periods of time.

Recently, the propensity of chemicals to form residues that produce harmful effects has become an important concern in toxicity predictions. These concerns are of two general types: 1) Accumulations that produce objectional flavor, and 2) acculumations, usually of persistent chemicals, that reach concentrations that are toxic to consumers of the organism bearing the accumulations.

Finally, another concern when predicting acceptable toxicant concentrations for aquatic organisms relates to the quality of the test animals. However, because that concern is relevant principally to the quality of data obtained, I will not consider it further in this discussion. Many more concerns could be identified, but these are sufficient to give a "feel" for the complexity involved in predicting toxicity.

Let us now focus on one of the proposed concepts for predicting acceptable concentrations--the application factor approach. Probably Hart, Doudoroff and Greenbank (1945) were among the first to suggest the use of application factors to (as they termed it) predict biologically safe concentrations. Even though aquatic toxicology was hardly born at the time they published, their concepts and perceptions are still very much "on the mark" and surprisingly current. Their approach included compensation for different sensitivities of various species, variable toxicity due to different receiving waters, and the effect of length of exposure. Their use of what in essence is the slope of the time-mortality curve to make inferences about cumulative toxicity is truly remarkable considering the embryonic state of aquatic toxicology at that time. They clearly cautioned workers about toxicological consequences resulting from reactions of the toxicant with various water constituents.

In subsequent papers, workers in the field, especially Doudoroff, frequently discussed the need to lower LC50 values in order to arrive at a safe exposure concentration. Times were such, and aquatic toxicology was so embryonic and unrecognized, that no one ventured opinions about how much reduction was needed at that time.

Henderson (1957) in the first of three seminars on "Biological Problems in Water Pollution," ventured forth with a reduction of 1/10 of the 96-hour

TLm as a "tentatively suggested" value. He considered this a "sizeable" reduction and even dared then to illustrate with an example in which he arrived at a factor of 1/12!

These and subsequent papers all stayed principally with arbitrary values and generally did not suggest how values might be derived experimentally, although Hart et al. (1945) did use experimental data as a part of their proposed formula.

Warren and Doudoroff (1958) may have been the first to propose experimentally derived application factors. They used 30-day toxicity tests in artificial streams to determine application factors for pulp mill wastes.

Just after the powerful 1965 Water Quality Act was passed and just before the "environmental awakening," Mount and Stephan (1967) proposed a method of experimentally deriving an application factor (AF) for each toxicant. They suggested that if one divides the highest concentration tested, in which no adverse effects during a life cycle test were found by the 96-hour TLm, the fractional value might be characteristic of the toxicant and constant for most or all fish species and water types. While they did not so state, there appears to be a sound toxicological basis for expecting the ratio to be constant. Specifically, the mode of action of a given toxicant is similar for various species of fish, but the threshold concentrations producing the effects are different for various species, thus producing various species sensitivities. Since the stage in an organism's life history most sensitive to a toxicant will vary between species, then any consistency in the AF value is probably caused by chance rather than a predictable toxicological principle. It is probable that any consistency between AF values for other animals (such as invertebrates) and fish is unlikely since the mode of action is probably different.

Mammalian toxicologists have also used a similar predictive approach. For example, Hayes (1967) described a chronicity factor to characterize the cumulative toxicity of chemicals.

The following evaluation of AF's is based largely on data from the committee report cited previously. The opinions expressed, however, are mine and not those of the committee.

The objective in using an AF approach is to integrate effects of variable species sensitivity, length of exposure and effect of water characteristics on toxicity, and to enable one to estimate acceptable concentrations without long expensive tests on a large number of species and waters. If the 96-hour LC50 divided into the MATC* is a reasonably constant value for most fish species, then the AF multiplied by the LC50 for any species of fish in any water type would estimate the acceptable concentrations for that species. The data in Table 1 are summarized from Andrew et al. (1977). The quotient of extremes for the MATC is found by dividing the highest concentration in a set of lowest concentrations just producing an effect by the lowest concentration in a set of highest concentrations not producing an effect. The quotient of extremes for the AF is calculated by dividing the largest numerical value of the AF by the smallest value in a set of values. In both cases, a set can include only one toxicant, since the AF value is expected to be different for different toxicants.

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*Maximum concentration that caused no significant effect on the reproduction, growth, and survival of test animals during a full life cycle.

TABLE 1. VARIABILITY OF MATC AND AF VALUES

Toxicant	Number of Tests	Quotient of Extreme Limits	
		MATC	AF
Atrazine	3	8.0	5.8
Cadmium	3	20	5.3
Chromium (IV)	2	20	35
Copper	6	14	13
Diazinon	2	28	136
Lead	2	4.0	2.4
Lindane	3	2.7	5.0
Malathion	3	161	3.5
Methylmercury	3	13	17
Zinc	4	46	206

If one compares the quotient values in the columns for MATC's and AF's for each toxicant, one finds that--among the ten toxicants for which data are given involving 31 chronic tests--the AF has less variability in five and the MATC has less variability in five. However, six of the ten values for AF's and MATC's are not significantly different.

These comparisons suggest rather convincingly (given our present ability to measure MATC and AF values) that one gains no more accuracy in estimating acceptable safe exposure concentrations by using an LC50 and an application factor than if one simply selects an MATC and uses that value for all fish species.

Obviously, in nearly every instance the true MATC will be lower than the LC50. If MATC's for only one or a few species are known, then using that value as an acceptable concentration probably will result in the selection of a lethal concentration for especially sensitive species for some portion of toxicants. Prudence certainly dictates that the acceptable concentration should be set below the MATC by some margin to protect us from our ignorance.

In the absence of any chronic data, but when a prediction of an acceptable concentration must be made, arbitrary reductions below the LC50's should be made. The amount of reduction can be derived by generalizing from the ratios of MATC's to LC50's for many substances chemically similar, or can be based on an average value representative of all toxicants for which such data are available. In other words, I'm suggesting that even though the present data base does not show AF's to be constant values, for arbitrary estimates they are as good as any other bases.

Many practical experimental problems can reduce reproducibility and make the concept appear invalid when it is not. Certainly our inability to measure biologically active forms of the toxicant can produce vast errors. Few data exist for judging the reproducibility of the MATC. We know that LC50 values vary substantially. These two sources of error can, and undoubtedly sometimes do, cancel or supplement each other to produce more experimental error.

On the other hand, data are accumulating, as for example McKim and Benoit (1971), to show that different species differ in their most sensitive stages. As stated earlier, this would seem to undermine the toxicological basis for the AF concept as proposed by Mount and Stephan (1967).

I began this paper by emphasizing the need to predict toxicity with minimum effort and maximum speed, and now I have ventured an opinion that the most commonly used predictive method is not supported by the data base. What, then, is an alternative?

While far from desirable, we can see that the use of a single MATC as the acceptable concentration is at least as good as the AF. Both the cough test (Drummond 1977) and the embryo larval test (Macek 1977; McKim 1977) offer promise as more accurate and non-arbitrary methods to estimate acceptable concentrations for a variety of situations. Given demands of present legislation, no research need is greater than the development of rapid and accurate screening methods to estimate toxicity. I am firmly convinced, however, that we must continue to use the life-cycle chronic test as our laboratory guidepost to assess the suitability of rapid screening tests. Without a solid chronic toxicity data base, we will be unable to judge the value of any other method to predict chronic toxicity. In the last 5-10 years, the chronic toxicity data base has increased many fold and provides an understanding that should be helpful in our search for better predictive methods. Field monitoring should be used to assess our overall ability to predict effects resulting from the use of our predictions but not for initially measuring acceptable concentrations.

The present need for establishing biologically acceptable concentrations of as many as 1500 new products each year, makes crystal clear that our past pace of data generation will have to be increased two to three orders of magnitude. Either more resources must be obtained or else a faster means to produce data must be found. Probably no method will always be correct, and we may have to be content with being right "most of the time." Perhaps never before have we faced a challenge so important to our national welfare as the one produced by the information needs of the Toxic Substances Control Act. Since the consequences of being unnecessarily restrictive are different, but perhaps as severe as being too liberal, our best effort will be none too good.

In conclusion, the biological validity of the AF concept certainly is not yet disproven, but the present data base is such that even if the concept is biologically valid, the practical problems involved in the determination of AF's make the approach of questionable utility. Furthermore, the present data base implies that the MATC of one species will provide with greater ease an equally accurate estimate of an acceptable concentration for other species if a safety factor is also applied. In view of current needs, we must rapidly improve our ability to predict acceptable concentrations for aquatic organisms.

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CLOSING REMARKS--AN OLD FROG CROAKS AN APPEAL FOR LOGIC

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First, I want to express to the sponsors--the Department of Fisheries and Wildlife of Oregon State University and the United States Environmental Protection Agency--my deep appreciation of the honor that has been accorded me by the dedication of this symposium. To the head of my department, Dr. Richard Tubb, who conceived this means of recognition of my services to the department and my profession and who has worked diligently toward its successful realization as a very special and memorable occasion at the time of my retirement, and also to Mrs. Alma Rogers, who assisted him with the arrangements, go my particular thanks. Also, to all the participants who have taken the trouble to prepare papers for presentation here--contributions that have been of great interest to me--and all those who have traveled long distances to attend this symposium or have written to me to extend their greetings, I am truly grateful. The cosponsorship of the symposium by EPA is signally gratifying to me. Though never an employee of EPA--sometimes even its opponent in adversary proceedings--I have felt since its inception as though I were a kind of honorary member. My many years as a water pollution biologist with the U.S. Public Health Service and the encouraging support and many courtesies extended to me by my former associates and other friends in EPA laboratories, and by the Agency, have generated this special feeling of affinity or fellowship, although I retired more than 11 years ago from the federal government. To A. F. (Fritz) Bartsch, to Donald Mount, and to Clarence Tarzwell (recently retired), I am particularly indebted in this connection.

Because I have some highly critical remarks to make today about one particular EPA publication, I want to make it very clear that I have great respect for my many competent and dedicated colleagues in EPA and for their notable research accomplishments. In no way can I hold them responsible for the defects of the report in question, and I wish to fault nobody except its anonymous authors in the Criteria and Standards Division, Office of Water Planning and Standards. I well realize that in our overgrown federal bureaucracy, monster agencies such as EPA can be many-headed like Hydra, with one head often not knowing what another one knows, does, or thinks, and not bothering to ask or to listen carefully. I am sure that some of my friends in EPA are or will be as unhappy as I am with some of their organization's products, the quality of which they had no power to control. They may welcome my saying more emphatically than they would want to say what they too have been thinking.

What have been my thoughts concerning my career as the time of my retirement approached? Naturally, I wish that my contributions to water pollution biology and environmental protection were as important and influential as some of my friends have tried to assure me they have been. Long ago, I believed that they would be. I started out as a smallish frog in a little pond disdained and shunned by smarter frogs. It was the early 40's, when water pollution control was primitive and my colleagues who were making significant contributions to water pollution biology in the United States could be counted on the fingers of my hands, or even on one hand. One did not have to be great to be one of the top frogs in my unattractive puddle. My early efforts to refine and standardize toxicity bioassays and to promote their use in waste disposal control seemed well worthwhile and were soon widely approved. Although I did little more than expedite inevitable developments, the widespread adoption of the recommended bioassay methods in this country and abroad was gratifying. My critical review of much of the limited available literature in the field and my performance of a few simple, carefully designed experiments soon made me an unchallenged expert. I moved from Cincinnati to Corvallis in 1953 at Professor R. E. Dimick's invitation. I was to develop, with Charles Warren and others, an OSU-PHS cooperative research program. As our joint research facilities and staff grew and improved rapidly, the opportunities to make important contributions seemed greatly enhanced. The need for a more aggressive attack on water pollution was evidently being recognized. I thought that a rational plan of development of our pertinent--although admittedly still very limited--ecological, chemical, and toxicological knowledge, and an equally rational system of its regulatory application would soon be designed and agreed upon by those in charge of the effort. I was eager and ready to be one of those leading the way, proud of our expanding laboratory complex here, which became a little Mecca for the still small number of water pollution biologists. But then came the flood, the unprecedented rapid expansion in the middle to late 60's, of environmental protection activities in our country. I had become a bigger frog in a pond somewhat enlarged by some busy beavers, but my pond now suddenly became a large lake, whose often turbulent waters were soon invaded by frogs coming from many other pools with all kinds of conflicting opinions. My influence there consequently waned; it is now almost negligible, in spite of my continued, sometimes frantic activity.

Impressed with signs of my apparent success and importance, such as the extent of my travel and the size of my consulting fees, in recent years my late brother Michael, the distinguished microbiologist, was no longer calling me a "sewage worker." (This appellation he had gleefully assigned to me long ago when he found me perusing the Sewage Works Journal, an early predecessor of the Journal of the Water Pollution Control Federation.) Environmental protection became a well-respected, well-funded, enthusiastically acclaimed field of endeavor. However, I was not very pleased, for its too rapid, almost chaotic development has not been conducive to careful discrimination between fact and fancy, right and wrong, sense and nonsense. Now that my pretension of outstanding intellectual leadership can no longer be maintained, I am just another frog contributing to a discordant chorus by croaking my discontent. Now is a good time for me to retire completely from the fray. But, speaking out on controversial issues in defense of rational positions, no matter how futile it may be, is a habit difficult for me to break.

What future do I now see for aquatic toxicologists and aquatic biologists in general in the field of water pollution control? I must say frankly that I am not very optimistic. I see much bitter disappointment and frustration for those competent, dedicated, and perceptive investigators who, like myself, would like to see the results of their research promptly and intelligently applied by the regulatory agencies. I see continued expenditure of much talent, money, and effort on research of high quality that leads to no visible, practical benefits, except perhaps, in the distant future. We can hope, of course, that some day things will be different, the administration of environmental protection laws will become entirely rational and truly scientific, and incompetence, superficiality, and disregard for the elementary principles of logic in the application of our research results will no longer be tolerated. Encouraged by this hope, or simply driven by intellectual curiosity, many of my younger colleagues doubtless will continue to exert their best efforts in seeking to advance knowledge in our field. But the value of their most significant factual contributions and most pregnant new ideas--even ideas that are not very profound or difficult to understand--they should not expect to be soon recognized except by a small number of colleagues also engaged in research. They should not assume that administrative (regulatory) decisions on which these contributions and ideas obviously have a direct bearing will be influenced and adjusted correctly to reflect the new knowledge.

Why do I hold this pessimistic view? Well, let me give an example of the kinds of frustration that I have recently experienced. My disappointment was not unique, but it was somewhat more distressing and humiliating than most of the others of its kind. And, it should be remembered that I am far from being a beginner in my field; my views and contribution should not be quite as easily ignored as those of numerous younger colleagues.

Last month, I examined a new publication just released by EPA (U.S. Environmental Protection Agency 1976), a 510-page document entitled "Quality Criteria for Water", a copy of which had kindly been supplied to me. Its perusal in part left me quite shaken. The formulation of sound water quality criteria pertaining to the protection of aquatic life and fisheries has been my predominant interest or objective during most of the last 35 years. With that end in view, I have done much thinking and have conducted intensive experimental and literature research in the toxicology of the simple and complex cyanides, the dissolved oxygen requirements of fishes, and other such matters. Naturally, I want to know to what extent the water quality criteria being proposed or used in water pollution control and the current regulatory practices are being influenced by my efforts and recommendations. So, it was with much interest that I began to examine the document presenting water quality criteria now being recommended by EPA, that powerful government agency charged with the administration of federal water pollution control legislation.

First, I looked at the section dealing with cyanides. As some of you know, I have been able to demonstrate quite conclusively, with the invaluable assistance of student and faculty colleagues, that the "total cyanide" concentration in water containing complex cyanides is toxicologically almost meaningless (Doudoroff 1956; Doudoroff, Leduc, and Schneider 1966; Doudoroff 1976). The toxicity to bluegills, for example, of acutely toxic cyanide solutions with total cyanide concentrations as low as 1 or 2 mg/l or less is determined entirely, or almost entirely, by the concentrations of free cyanide or, more specifically, of molecular (un-ionized) hydrocyanic acid, HCN. This relationship is usually true of the toxicity of much more concentrated solutions also, but there are known exceptions. At the pH of most natural waters, most of the free cyanide (molecular HCN plus the CN^- ion) is present as HCN, the more toxic of the two forms of free cyanide (i.e., more toxic than the CN^- ion), therefore, the distinction between HCN and free cyanide is of little practical importance. The level of one can be easily calculated from that of the other when the pH is known. Undissociated metallocyanide complex anions, which can be much more abundant than free cyanide in cyanide-bearing wastes and polluted waters, are much less toxic than HCN, or virtually nontoxic. For these reasons, it seemed obvious to me that an entirely sound, basic, chemical water quality criterion pertaining to the suitability of cyanide-polluted waters for aquatic life has to be expressed as a concentration of free cyanide or of molecular HCN, and not of total cyanide. A reliable and sensitive chemical analytical method that distinguishes between the highly toxic and relatively harmless or toxicologically inactive forms of cyanide clearly was needed, I told my colleagues long ago. Largely because of my early findings and urging, several quite satisfactory methods for determination of molecular HCN have been developed by my associates at Oregon State University (Schneider and Freund 1962; Claeys and Freund 1968; Broderius 1973) and by other American and British investigators (see Doudoroff 1976, pp. 9-10). Some of these methods were used in confirming the toxicological conclusions stated above. Thus, through intensive research, the technical problem to which I had addressed myself was essentially solved, and I was very well pleased indeed with the accomplishment, which seemed to call and point the way for much more research of the same general kind.

But what did I find in the EPA report? I found that the great toxicity of HCN is duly noted, as is the fact that the ratio of HCN to total cyanide in waters polluted with cyanides is highly variable, depending not only on the nature of the cyanide compounds introduced but also on the pH, illumination, and other conditions. In addition, I found this poorly worded but nevertheless devastating statement (p. 132): "Since such chemical and physical conditions will dictate the form of cyanide, the cyanide criteria _must_ be based on the concentration of _total_ cyanide present in the water" (emphasis added). Accordingly, a cyanide concentration limit of 5 µg/l (0.005 mg/l) is recommended as a criterion for aquatic life without specifying that this amount of cyanide must be free or present as molecular HCN.

Is the quoted conclusion a logical one? Apparently, the authors of the report think that it obviously is; they make no effort to justify or defend their assertion, although it flatly contradicts the published recommendation of the National Academy of Sciences and National Academy of Engineering

(1973), which I helped to prepare. Well, if that conclusion is accepted as reasonable, then corresponding conclusions surely must be reached also with respect to ammonia, sulfides, heavy metals, and other toxicants. It is well known that the ratios of highly toxic molecular (un-ionized) ammonia and hydrogen sulfide to total ammonia and total sulfide, respectively, in polluted waters vary widely, depending on such factors as pH, temperature, and ionic strength, and that their variation is toxicologically important. Thus, if the EPA authors were at all consistent in applying the questionable reasoning on which the statement quoted above is based, they should certainly have concluded that, since chemical and physical conditions dictate the forms of ammonia and sulfide, the ammonia and sulfide criteria must be based on the concentrations of total ammonia and sulfide present in the water. But what actually are the ammonia and sulfide criteria recommended by them? The criterion for ammonia (p. 16) is 0.02 mg/l of un-ionized ammonia only (not total ammonia, for which no limit is proposed), and the sulfide criterion (p. 410) is 2 μg/l of undissociated H_2S only (not total sulfide or total dissolved sulfide). Evidently, the authors concluded that, since chemical and physical conditions dictate the forms of ammonia and sulfide, the ammonia and sulfide criteria must be based on the concentrations of molecular NH_3 and H_2S only, disregarding the less harmful or relatively nontoxic NH_4^+ and HS^- ions.

What can be the reason for the obvious inconsistency? There can hardly be any nice, logical justification. The only explanation that I can suggest, other than sheer, negligent incompetence or dishonesty of the authors, is that logic has gone out of style and consistency is no longer highly valued in our field of environmental protection. Now, appeals to emotion and prejudice prevail all too often over sound arguments, and a host of confused "experts" have sprung up almost overnight like mushrooms. Immutable laws of chemistry and physics dictate the transmutations of cyanide and ammonia, but the choice of the water quality criteria evidently has been dictated only by whim or caprice. Capriciously, the results of thorough, painstaking research into the toxicology of the complex metallocyanides and careful development of needed analytical methods that have made possible the establishment of sound cyanide criteria like those previously developed for ammonia are totally ignored--not even mentioned--in the EPA publication. They have been brushed aside and made to seem irrelevant with a single, flat assertion that sounds like a statement of an indisputable corollary of some natural law, but which actually is groundless and contrary to reason. If this assertion were true, there would be no good reason, of course, further to test or simplify the new analytical methods for determination of HCN.

The possibility that a harmless form of cyanide present in water will be soon converted, under certain conditions, into a highly toxic form should not be overlooked in controlling water pollution. However, only after this transmutation has actually occurred, a fact now readily demonstrable by chemical analysis, is the suitability of the water as a medium for aquatic life affected and it may or may not occur effectively. Photodecomposition of nontoxic iron-cyanide complexes, for example, may be negligible in deep, turbid, or shaded waters, and slowly liberated cyanide may decay or escape as rapidly as it is released, free cyanide not being a presistent pollutant. A large biochemical oxygen demand (BOD) of an effluent or receiving water is worthy of attention, but the dissolved oxygen concentration (DO) is a much more meaningful index of the suitability of polluted waters for aquatic life (except for some decomposers) than is the BOD. When reaeration is rapid, an initially very large oxygen demand may be gradually satisfied without causing any harmful depression of DO. It has long been generally recognized, therefore, that sound water quality criteria for the protection of aquatic life against the oxygen-depleting effects of putrescible organic wastes must be appropriate limits of DO and not of BOD. In what fundamental way is the problem presented by the potential toxicity of nontoxic, complexed cyanide different from that presented by the oxygen-depleting potential of organic wastes? I can see no difference requiring diametrically different approaches to the two problems.

Because of EPA's prestige and power, its ill-considered pronouncements can block technical advances for years. Recently I have presented extensive testimony in the State of Illinois in support of a proposal (by my clients, the Illinois Petroleum Council, and others) that a free cyanide standard of water quality be substituted for an outdated total cyanide standard that had long been in force in that state. I hoped soon to see wide approval of such improvement of standards by state regulatory agencies and I strove to bring it about. But having seen the EPA report stating flatly that pertinent water quality criteria "must be based on the concentration of total cyanide" and implying that each recommendation contained in the report represents a consensus or majority opinion of experts based on the latest available scientific information, I now see almost no possibility of success. Although I do not believe that such matters are best settled by the adversary method, I now would like to see the issue litigated. Perhaps in a court of law, logic would prevail. I hope that some of my influential, reasonable, and well-informed friends in EPA will be willing and able to take some effective action leading to early correction of the mistake.

In the section of the report on cyanide, I found other statements in addition to the one quoted that are erroneous; some are incompatible (contradictory). These errors are not of critical import, however, so they need not be pointed out and discussed here. The treatment of the subject is generally inadequate, and I think that attribution of the content of the entire volume to "the efforts of many dedicated people" including "technical specialists throughout the Agency's operational programs and in its research laboratories" (p. ix) is not something that should greatly please competent members of the EPA research staff.

After examining the section on cyanide, I turned to that dealing with dissolved oxygen criteria--another subject of outstanding interest to me--and found it no less depressing. There is no relation or resemblance at all between the new EPA recommendations and the much more elaborate ones of Doudoroff and Shumway (1970) or those of the National Academy of Sciences and National Academy of Engineering (1973), which were based in large part on those of Doudoroff and Shumway. Those recommendations have been ignored. The DO criterion adopted by EPA is that proposed 40 years ago by Ellis (1937) for warm-water fish habitats, simply a minimum of 5 mg/l. Its recommended application has now been extended to all fresh waters, warm or cold, including interstitial waters of the gravels of salmonid spawning beds. Applicability of his criterion to cold-water fish habitats was not claimed by Ellis.

The EPA criterion is said to be based primarily on observations made in the field (mostly those of Ellis and his associates) on the relation between observed DO levels in various sampled waters and the variety of fishes found there; the presence of a "well-rounded fish population" was taken as an indication of satisfactory conditions.

The deficiencies of the evidence on which Ellis' conclusions were based, that is, reasons for its unreliability, have been fully discussed by Doudoroff and Shumway (1970, pp. 241-247). Their carefully developed argument and the suppporting data, not mentioned by the EPA authors, cannot be adequately summarized here. It was shown that good, mixed fish faunas, as defined by Ellis, actually can occur in waters where DO levels do not exceed 4 mg/l for very long periods, are often below 3 mg/l, and sometimes are as low as 1.4 mg/l or less. These results do not prove, of course, that fish production is not seriously impaired at such low DO levels. Neither does the observation, cited in the EPA report, that rainbow trout thrive in Lake Titicaca, where, because of the altitude, DO does not exceed 5 mg/l, signify that trout production is not reduced materially by reduction of DO to 5 mg/l in other waters with much higher natural DO levels.

I was amused by the statement in the EPA report that, in seeking to relate fish abundance and distribution to DO in the field, "enough observations have been made under a variety of conditions that the importance of oxygen concentration seems clear." I cannot quarrel with that statement, but is the mere demonstration of the importance of an environmental factor sufficient for the establishment of a water quality criterion? The pertinent experimental data, most of which have been thoroughly and critically reviewed by Doudoroff and Shumway (1970), also show very clearly the importance of DO. Why, then, has the vast amount of such information obtained during the past 40 years, in our laboratories and others, been mostly disregarded by the EPA authors? Quite disturbing to me was this justification given by them of their reliance predominantly or almost entirely on data from the field: "The requirement that the data be applicable to naturally occurring populations imposes limits on the types of research that can be used as a basis for the criterion. Aside from a few papers on feeding, growth, and survival in relation to oxygen concentration, very little of the laboratory based literature has a direct bearing; field data are in general more useful."

How many of the other water quality criteria, that have been recommended in the same publication as defensible criteria pertaining to the requirements of aquatic life (mostly criteria for toxic pollutants) are based predominantly on field data? How many, I should ask, are based on any data other than data from laboratory experiments? Not many, I am sure. What is the cyanide criterion based on, for example? Only on laboratory data, and particularly on observed effects of 10 μg/l of free cyanide on the swimming performance of salmonid fish. Actually, the vast amount of experimental (mostly laboratory) data bearing on the DO requirements of fishes that is now available (data on effects of DO reductions on survival, development, feeding, growth, fecundity, swimming ability, behavior, respiration, and oxygen consumption) is a basis for water quality criteria that is far more satisfactory than the bases for most of the other recommended criteria. By contrast, the available data from field studies on fish distribution and abundance (natural fish populations) in relation to DO are still extremely limited, and their usefulness in the verification or refinement of DO criteria is almost negligible. Again, logic seems to have been abandoned. If the extensive data from laboratory studies are indeed of almost no value or pertinence to the formulation of DO criteria, does it not follow that there are no adequate bases at all for most of the other water quality criteria pertaining to aquatic life that have been advanced? Should not these other recommended criteria have been withheld (not published) for lack of sufficient foundations?

I myself have been urging other investigators to pay more attention to natural conditions and to their simulation (especially with regard to bioenergetic considerations) in the design of experiments directed toward better understanding of the effects of water pollution on aquatic life (Doudoroff 1977; Doudoroff and Shumway 1970). I know that fish, in their natural habitats, are not usually exposed throughout their life cycles, or for very long periods, to nearly constant concentrations of pollutants, or to unlimited amounts of food obtainable almost without effort, or to an artificially restricted food supply. I have repeatedly pointed out that interference with reproduction in polluted waters of limited extent can be often fully compensated for by increased growth rates (due to reduced competition for food) or by the immigration of young from contiguous waters. I believe that some of our water quality criteria based on results of unrealistic experiments may be misleading, and some regulatory water quality standards directly derived from them can be entirely too restrictive, particularly when the criteria derive from life-cycle tests at constant concentrations of toxicants. But I certainly would not go so far as to say that the experimental work of the past has provided little useful information. I do not propose that we abandon our laboratories and all take to the field to sample various polluted waters and their fish populations in order to arrive at the best water quality criteria.

My impression is that, in the eyes of the authors of the EPA report, the intensive experimental work on the DO requirements of fish and the chemistry and toxicology of the complex cyanides that my co-workers and I have done over the years has been almost completely wasted effort. Certainly, their recommended water quality criteria would not have been any different had none of this work ever been done. One may well be impelled to ask if it is not a pity that so much time and money were spent so unproductively, because of my poor judgment. And is not Gary Chapman of EPA, who spoke to us about the different forms of copper and their relative toxicity, perhaps largely wasting his time also when concerning himself with such matters? If water quality criteria for copper must, for some reason, be "based on" total copper, no matter how successfully the toxic forms may be identified, their interactions described, and analytical methods for their separate determination developed, the subject of Chapman's report can be of academic interest only. Perhaps he too should be out in the field collecting and identifying fish. Has William Spoor also been wasting federal government money in Duluth by studying effects of DO reduction on fish development?

I must say that I have not always felt that my efforts have been unappreciated or that my recommendations relative to water quality criteria have been ignored. On the contrary, I have been often gratified by the attention given to my findings and conclusions by my most respected professional colleagues, including leading EPA biologists. The honor accorded me at this time clearly bespeaks abundant appreciation of my modest accomplishments. And the authors of the important, recent publication "Water Quality Criteria 1972", the socalled "Blue Book", prepared for EPA by the National Academy of Sciences and National Academy of Engineering (1973), having given me a courteous and attentive hearing at no expense to me, accepted in large part those of my views that were presented to them. As noted already, the cyanide criteria recommended by that prestigious group are concentration limits of free cyanide, not total cyanide. The DO criteria recommended, although not entirely in agreement with the recommendations that I presented and defended, did reflect my views in large degree, and I felt that their adoption was an important step in the right direction. The adoption of graded criteria of water quality appropriate to different "levels of protection" of aquatic life (to be selected on the basis of socio-economic considerations), which were recommended in dealing with pH and with suspended and settleable solids as well as with DO, was most gratifying, because it had been first proposed and strongly advocated by me.

Unfortunately, some important inconsistencies or illogical features similar to those of the recommendations in the new EPA report mar also the recommendations presented in the "Blue Book". At least one of the modifications made of the proposed DO criteria of Doudoroff and Shumway (1970) and their related recommendations was not, in my opinion, justifiable; that change, an incongruous kind of hybridization of old and new approaches, clearly was adopted as a compromise because of reluctance of some of the authors to depart entirely from precedents. Some serious errors and inconsistencies are to be expected in a work prepared in the manner and short time in which the "Blue Book" was prepared. But it seems to me that in the course of the preparation of the new EPA publication, on which work has been going on for a long time, the inconsistencies and other mistakes to be found in the somewhat too hastily prepared "Blue Book" should have been largely corrected or avoided, not multiplied or aggravated.

The 1976 report is not the first such report prepared by EPA. This new volume is a revision of proposed EPA Water Quality Criteria, presented in a publication that was not widely distributed but whose limited availability was announced by means of a notice published in October, 1973, in the Federal Register (U. S. Environmental Protection Agency 1973a). It is noteworthy that the cyanide criteria proposed by EPA in the earlier (1973a) report are essentially identical with those recommended in the "Blue Book". The DO criteria proposed at that time are somewhat different from the "Blue Book" criteria, but were said in the Notice of Publication to be "generally consistent" with them. I may have seen these proposed DO criteria but cannot now recall examining them; a single DO level of 5 mg/l was certainly not given as a generally applicable water quality criterion. The disagreement between the most recently published cyanide and DO criteria recommended by EPA and those proposed in the "Blue Book" obviously are not attributable to inadvertence. Why the criteria initially proposed by EPA have now been rejected and different ones substituted, and who first proposed the drastic changes, I do not know. In the 1976 report, it is stated that the revision of the previously proposed criteria was "based on a consideration of comments received from other federal agencies, state agencies, special interest groups, and individual scientists." But it is not apparent that authors of the "Blue Book" and other leading experts had an opportunity to review and comment on all the new or revised criteria before publication, to object to proposed changes, and to explain their objections. I understand that "pre-publication" copies of "Quality Criteria for Water" were distributed in October or November of 1975 to a number of scientists or laboratories outside EPA for review. However, I do not know how many of these copies were distributed or to whom they were sent, and I have learned that the proposed DO criteria presented in those copies were still quite similar to the "Blue Book" criteria and those of Doudoroff and Shumway. Thus, it seems reasonable to suppose that nobody of the scientific community outside EPA was given the opportunity to examine and object to the finally published DO criterion and supporting statement; reviewers of the prepublication version had good reason to believe that the "Blue Book" recommendations would not be entirely ignored or contradicted in the published EPA report. I was never consulted nor asked my opinion of the new cyanide and DO criteria by EPA, although my pertinent expertise could hardly have been overlooked. Their publication was a complete surprise to me, like a bolt from the blue.

It has been suggested to me that the real reasons for the drastic revision of the original EPA criteria may perhaps have been political rather than scientific, having something to do with possible difficulties of enforcement of regulatory standards based on them. The suggestion was that the authors may have understood perfectly that the cyanide criteria can very well be "based on" reliably determinable free cyanide or HCN levels and that limits of free cyanide or HCN concentration are scientifically much sounder, more reliable criteria than limits of total cyanide concentration, but decided that acknowledgment of these scientific facts would be politically inexpedient or embarrassing. However, deliberate obfuscation or concealment of the truth obviously would have been intellectually dishonest, and I do not want to accuse anyone of intellectual dishonesty. The administrator of EPA had been directed by Congress to publish "criteria for water quality accurately reflecting the latest scientific knowledge" and not reflecting his staff's latest notions of how science or truth can best be twisted to achieve some practical objective. In preparing my critical comments, I assumed that the authors of the EPA report strove to fulfill this charge (as they implied they did) and so were not intentionally inconsistent and purposely misleading.

It is noteworthy that the authors of "toxic pollutant effluent standards" proposed by EPA about three years ago (U. S. Environmental Protection Agency 1973b) were aware of the importance of the distinction between free and complexed cyanide. My clients, the American Iron and Steel Institute, and many others objected to those proposed standards for various reasons, among which were terminological and methodological vagueness and errors. At a hearing in Washington, D.C., in 1974, I expounded extensively on the chemistry and toxicology of the cyanides, as did also my former student, Steven Broderius, at a later hearing. I had hoped that our efforts to clarify the complicated problems involved would lead to a better understanding by all those in EPA concerned with effluent and water quality standards and criteria. Because of the various objections raised, the proposed effluent standards, which had some sensible features and could have been improved enough with a few changes to make them fairly reasonable, were finally withdrawn by EPA.

But the water quality criterion for cyanide now being recommended by EPA suggests that understanding, if it has changed at all, has deteriorated. New proposals concerning regulatory standards could well be totally wrong. I am reminded again of the nature of Hydra, with which I have already drawn an analogy. When you chopped off a head that threatened you, you were worse off than before, because two more dangerous heads grew in its place.

I want to repeat, however, that my purpose here has not been to attack EPA, an organization to which I still feel, justifiably or unjustifiably, that I somehow belong. What I am really attacking is the shallow, careless, and irresponsible thinking that pervades the environmental protection movement. This irrationality is to be found outside EPA, in state regulatory agencies for example, probably at least as often as in the powerful federal agency; it is often to be found even in our universities, where we expect to find models of detached rationality. I am objecting to all indifferent tolerance in my profession of gross inconsistency, which betokens gross error, for it can exist only when there is such error. I am croaking an appeal for logic. If even old frogs like me refrain from raising their voices in protest, for fear of offending some other frogs in our lake, who will? To whom will the tadpoles in the lake be able to look for inspiring intellectual guidance? At this stage of my career, I have nothing to lose by being outspoken, and I am sure that many of you, as well as others, no matter where they work or seek support, will share my sentiments.

I thank you and wish you all a good year and successful researching through 1977.

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Dr. Charles E. Warren presented a paper on "The Interpretation of Laboratory Results." The manuscript was not available at the time of printing. Exclusion is not meant to imply any criticism of the paper or the presentation.